Evaluation of Dietary Probiotic mixtures to improve Physiology and Health of *L. rohita* through gut microbiome Modulation under Mimic Aquaculture conditions

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List of Acronym/abbreviations (alphabetically)

%	Percentage
%WG	Percentage weight gain
ATP	Adenosine triphosphate
CFU	Colony forming unit
CLSI	Clinical and laboratory institute
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acids
FAO	Food and agriculture organization
FCE	Feed conversion efficiency
FCR	Feed conversion ratio
GDP	Gross domestic product
GIT	Gastro intestinal tract
GRAS	Generally regarded as safes
HCT	Hematocrit
HGB	Hemoglobin
LAB	Lactic acid bacteria
MCH	Mean corpuscular hemoglobin.
MCHC	Mean corpuscular hemoglobin concentration.
MCV	Mean corpuscular volume
MRS	De Man, Rogosa and Sharpe agar
ºC	Degree centigrade
OD	Optical density
OGA	Oxytetracycline glucose agar
PCR	Polymerase chain reaction
RBCs	Red blood cells
Rpm	Revolutions per minutes
SGR	Specific growth rate
T1	*Geotrichum candidum* QAUGC01
T2	*Enterococcus faecium* QAUEF01
T3	*Enterococcus hirae* QAUEH01
T4	*Bacillus cereus* QAUBC02
T5	*Geotrichum candidum* QAUGC01 and *Enterococcus faecium* QAUEF01
T6	*Geotrichum candidum* QAUGC01 and *Enterococcus hirae* QAUEH01
T7	*Geotrichum candidum* QAUGC01 and *Bacillus cereus* QAUBC02
T8	Commercial Probiotic
T-RFLP	Terminal restriction fragment length polymorphism
WBC	White blood cells
µL	Microliter

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Ifra Ghori

Abstract

Aquaculture is one of the fastest growing industries providing a great deal of high quality dietary protein worldwide. The aim of the present study was to beneficial modulation of fish (*Labeo rohita*) gut microbial communities by potential probiotics in mimic aquaculture conditions. This biobased solution is the eco-friendly alternative approach for enhancing productivity and infection control. With the perspective of Pakistan, although aquaculture is in infancy, but in future it seems to be a source of safe food and profitable economy. The present study was executed in three phases, during the first phase of study, the morphological and biochemical characterization of the isolates from gut of *Labeo rohita* were examined, followed by identification of selected strains. All the tested isolates were catalase negative and oxidase negative. The identified strains were *Enterococcus hirae* QAUF01, *Bacillus cereus* QAUBC02, *Enterococcus faecium* QAUF18 and *E.mundtii* QAUF20. The *in vitro* analysis of selected isolates (*E. faecium* QAUF18, *E. mundtii* QAUF20, *B. cereus* QAUBC02, *E. faecium* QAUEF01, *E. hirae* QAUEH01, *G. candidum* QAUGC01, F7, F8, F19, F20, O1, O2, O14 and O29) and combination of selected identified bacterial strains with *G. candidum* QAUGC01, (co-culture of *E. faecium* QAUEF01 and *G. candidum* QAUGC01, co-culture of *E. hirae* QAUEH01 and *G. candidum* QAUGC01, co-culture of *B. cereus* QAUBC02 and *G. candidum* QAUGC01) was evaluated to check their efficacy as potential probiotic candidates. During the present study for the first time, co-culture of *Geotrichum candidum* QAUGC01 and bacterial strains were evaluated for synergistic probiotic characteristics. The studied parameters were acid tolerance, percentage survival in bile salts, hydrophobicity, antibiotic sensitivity and antibiotic resistance. The maximum adherence ability was observed in case of *G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture (40.35±3.45). The *B. cereus* QAUBC02 and its co-culture with *G. candidum* QAUGC01 showed more than 50 % tolerance to bile salts at different time intervals. Maximum tolerance to acid at pH 2 and 5 was observed by *E. faecium* QAUEF01. *G. candidum* QAUGC01 exhibited antimicrobial activity against selected pathogens. All the strains were sensitive to vacomycin and varied results were obtained for other antibiotics. In the second phase of the study, the selected probiotics were applied as a feed supplement in trial of 90 days to evaluate their impact on physiology of *L. rohita* . The treatments used were *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), combination of *G. candidum* QAUGC01 and *E. faecium*

QAUEF01 (T5), combination of *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), combination of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 and commercial probiotic (T8) were added to fish feed for ninety days . The control group was fed on basal diet (T0). All the probiotic treatments had significant impact on physiological parameters (growth, hematology, intestinal enzymes , body composition of the fish as compared to control fed group. Moreover, fishes fed on (T7) combination of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 significantly grew faster and showed improved hematology. However, higher protease activity in gastrointestinal content was observed in fishes fed on *G. candidum* QAUGC01 and *E. faecium* QAUEF01, whereas significant higher activity of amylase and cellulase was observed in group fed (T4) *B. cereus* QAUBC02. Probiotics also improved the blood profile in comparison to the control fishes fed on basal diet. Higher protein content was found in fish carcasses fed on *G. candidum* QAUGC01 while fishes fed on *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture showed significantly higher fat content. The fishes fed were challenged with *Staphylococcus aureus* survived as compared to control fed fishes which exhibited mortality after pathogen exposure.

The third phase was based on investigation of gut microbiology of *L. rohita* by culture dependent and culture independent techniques at the end of feeding trial. It was observed that feeding on probiotics had significantly alter the fish gut microbiology as compare to control fishes. Proteobacteria was dominant bacterial phylum in all treatments including control except *E. faecium* QAUEF01 that promoted dominance of Firmicutes. Fishes fed (T1) *G. candidum* QAUGC01 as single culture and (T7) *G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02 were dominated by *Galactomyces geotrichum* (30.18%). Higher reads of *G. candidum* QAUGC01 showed the survival and persistence capacity of *G. candidum* QAUGC01 in gut which consequently modulated the microbial communities. Furthermore, preliminary fish gut proteomic analysis revealed the presence of regulatory, stress and metabolic proteins produced by *G.candidum* QAUGC01. This work is the base line study for use of *G. candidum* QAUGC01 probiotic for commercial aquaculture based on the idea of probiotic based modulation of microbiome for healthy fish.

1. INTRODUCTION

1. INTRODUCTION

It is inevitable to promote high quality safe animal protein to gratify the ever rising needs of growing human population. Sea food though dominating worldwide has met with serious setbacks which are barring its progress (FAO, 2014). Major problems faced by aquaculture are poor growth, incomprehensive mortalities and diseases. The uncontrolled use of antibiotics and chemotherapeutic is leading to the emergence of antibiotic resistance which is negatively effecting both fish health and environment (Allameh *et al.*, 2015). These problems can be culminated by use of probiotics-a bio based substitute for customary practices (Bandyopadhyay *et al.*, 2015).“Probiotics are live microorganisms consisting either of single or mixture of strains that contribute to health of host provided they are taken in suitable amounts” (Rehaiem *et al.*, 2014). The multispecies/multistrain probiotic treatment may be considered more protective and more consistent than the monospecies probiotic treatment due to their synergistic properties (Timmerman *et al.*, 2004). Probiotics action is mediated by various modes including competitive exclusion, antimicrobial production, siderophores, organic acids and bacteriolytic enzyme production (Miyanaga *et al.*, 2011b). The spores of *Bacillus toyoi* as probiotics was initially used in Japanese eel’s commercial diet as feed additive which increased its growth and decreased the mortality (Kozasa, 1986). Probiotics modulate gut microbiota by colonizing in intestine, excluding pathogens from attachment sites, surviving in acidic environment and bile and producing digestive enzymes (Mukherjee & Ghosh, 2016). Aquaculture based products nowadays are favored option by consumers due to its high nutrient value especially essential fatty acids, vitamins and health claims as compared to livestock (Klaenhammer & Kullen, 1999). According to an estimate it is serving 20 to 60% world nutritional protein requirement and producing more than 50% of fish supply worldwide. The income generated by this activities are about US$ 130 billion.

Fish being poikilothermic is subjected to the variation of external environment just after hatching. The digestive tract at this stage though partially developed yet it starts feeding. Due to the vulnerabilities of the environment it can succumb to variety of pathogens. The application of probiotics at this stage shows more pronounced impacts than in their later life stages especially in terms of growth and immunity (Picchietti *et al.*, 2007).

Last two decades has observed a gigantic increase in aquaculture (FAO, 2014). Aquaculture in Pakistan it is still in beginning phase. Carps are cultivated at larger scale in Punjab, Sindh and Khyber Pakhtoon Khwa. *Catla catla, L. rohita* and *Cirrhinus mrigala* are indigenous fish species of Pakistan are of utmost importance with regard to economy of Pakistan. Natural water body of Pakistan comprises 193 different species of fish. 31 species are of market value providing unique dietary benefits such as proteins, minerals and important nutrients which is not accomplished by any other dietary source. In view of production, carp (*catla and rohu*) is still most significant specie of aquaculture that contribute nearly 72% in total freshwater production (Kühlwein *et al.*, 2014). Therefore proper management of aquaculture sector is required for its development.

The probiotics which are most frequently used are Lactic acid bacteria such as *Lactobacillus (acidophilus, brevis, fermentum), Streptococcus, Bifidobacterium, Lactococcus* and *Pediococcus* (Rehaiem *et al.*, 2014). Some species of yeast are also used as probiotics (Soccol *et al.*, 2010). These strains are commonly observed as safe and usually are food grade (Singh *et al.*, 2012). Lactic acid bacteria attached with intestinal mucosa thus preventing pathogens to establish there (Kim & Austin, 2006; Korkea-aho *et al.*, 2012; Mahdhi *et al.*, 2012). Initially probiotics are used as feed supplements in pig, cattle and poultry but later on it was applied in aquaculture practices (Tukmechi *et al.*, 2007).

Application of probiotics can modulate gut microflora affecting their physiology, digestion, health, growth, reproduction capacity, immunity prevents the host from getting infectious disease. It is well documented that probiotics improve growth by improving feed digestibility, specific growth rate, percentage weight gain and protein efficiency ratio, producing digestive enzymes which not only aid in digestion but also release growth factors such as vitamins, amino acids and fatty acids (Balcázar *et al.*, 2006; Dimitroglou *et al.*, 2011; Tukmechi *et al.*, 2007). Previously conducted studies in fishes indicated that probiotics can modulate immune system by elevating the levels of phagocytes, lysozyme, complement, respiratory burst activity and certain cytokines (Nayak, 2010). The complete hematology indicates the stressful and pathological conditions (Duthie & Tort, 1985). These parameters are age, health and specie specific (Hrubec *et al.*, 2001). It is investigated that the probiotics added diet in certain fishes enhanced hemoglobin, mean corpuscular volume, hemoglobin concentration and mean

corpuscular hemoglobin concentrations (Kumar *et al.*, 2008). The symbiotic relation of gut microbiome with its host is involved in disease resistance, host metabolism, digestion and immunity activation (Hooper & Macpherson, 2010; Kaiko & Stappenbeck, 2014; Lee & Hase, 2014).

Gastrointestinal tract bacteria are divided into autochthonous bacteria that are colonized on epithelium layer and allochthonous bacteria which are transient ones (Ringø & Olsen, 1999). Fish gut biodiversity is the myriad of commensal, pathogenic and symbiotic interrelationships (Merrifield *et al.*, 2010). Microbial communities of gut are decided by multiple factors such as pH, season, salinity, temperature, trophic level and feed (Nayak, 2010; Sullam *et al.*, 2012). Understanding of complex Gastrointestinal tract microbiota is important for designing effective strategies to manipulate them to increase animal welfare in terms of health and productivity (Romero *et al.*, 2014). Apart from bacteria fish gut microbiota comprises of yeast, viruses, archaeans and protozoans occupying specific biological functions. The most frequently found phyla in found in different fishes are Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria each occupying an important niche in digestive tract. The gastrointestinal tract is nutrient laden source for inhabiting microbiota (Brett, 1979; Saha *et al.*, 2006). It was investigated that Lactic acid bacteria, the slow growers represents a small proportion of intestinal microflora (Ringø & Gatesoupe, 1998; Verschuere *et al.*, 2000). Yeasts constitute the major microflora of healthy fishes. They produce numerous metabolic products due to their immense diversity in different species (Gatesoupe, 2007). Yeast found in fish intestine is divisible into two groups Ascomycota and Basidiomycota, *Saccharomycetaceae* is the most important family of Ascomycota genus *Rhodotorula* is the most dominant genus in marine as well as fresh water fishes belong to Basidiomycota (Chiu *et al.*, 2010; Harikrishnan *et al.*, 2010a; Tukmechi *et al.*, 2011; Welker *et al.*, 2007).

Gut microbiology has been studied extensively by culture dependent as well as culture independent approaches. Culture based techniques though economical represents incomplete microbial diversity prompted the scientists towards advanced molecular techniques for in-depth information (Eckburg *et al.*, 2005; Guarner & Malagelada, 2003). Modern molecular techniques namely PCR cloning and DNA sequencing, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), metagenomics/pyro sequencing have been successfully

interpreting the complexity of gut microbiology (Li *et al.*, 2012; X. Li *et al.*, 2012; Roeselers *et al.*, 2011). 454/Roche Pyrosequencing and Illumina technologies are most frequently used to study the fish gut microbiome. Illumine Hiseq 2000 was used to study the impact of feed composition and its abundance on the function and composition of gut micro flora (Ni *et al.*, 2014).

The main challenges of aquaculture are low productivity, control of infectious diseases and cost effectiveness. Conventional methods for used to promote growth and control of pathogens were antibiotics, chemotherapeutic and vaccine which lead to antibiotic resistance which is very dangerous both for environment and man. Probiotics can be integrated bio based solution for better growth, disease control, cost effective feed, maintenance of water quality and safe consumer friendly food.

OBJECTIVES

1. To evaluate the probiotic potential of selected bacterial and fungal strains.
2. To deduce the effect of probiotic feeding on growth and hematological indices of *Labeo rohita*.
3. To study the impact of probiotics on *L. rohita* gut microbial diversity.

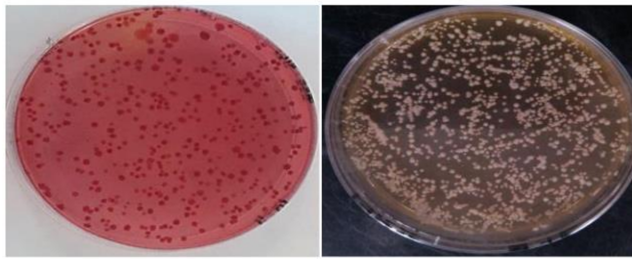
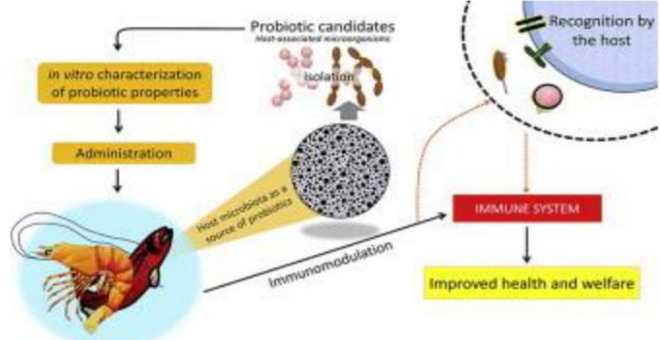
RESEARCH WORK PLAN	
Phase -I **Isolation, characterization and screening of strains for probiotic potential** 	➢ **Isolation, morphological and biochemical study of isolates** i. Gram staining ii. Catalase test iii. Oxidase test ➢ **Probiotic screening** i. Acid tolerance(Singhal *et al*.,2010) ii. Hydrophobicity(Magaldi *et al.*, 2004) iii. Bile tolerance (Walker and Gilliland,1993) iv. Antimicrobial activity(Magaldi *et al.*,2004) v. Antibiotic sensitivity(Bauer *et al*.,1966)
Phase-II **Application of selected probiotics in *Labeo rohita.***  	➢ **Selection of animal model (*Labeo rohita*)** ➢ **Growth parameter** i. (% Weight gain, Feed conversion ratio, Feed conversion efficiency and Specific growth rate ➢ **Hematology** i. (RBCs, WBCs, Hemoglobin, Mean corpuscular hemoglobin, Hematocrit, Platelets, Lymphocytes, blood glucose level) ii. Proximate Analysis(Crude Protein (Kjeldahl method) , crude fat (Soxthlet apparatus) and ash content ➢ **Enzyme analysis** i. Protease (Tsuchida *et al*.,1986) ii. Amylase (Bernfeld,1955) iii. Cellulase (Denison & Koehn, 1977)
Phase-III **Study of Fish gut microbiology & Proteomics**	➢ Culture dependent technique(CFU/g) ➢ Culture independent technique (Metagenomics) ➢ Proteomics (MALDI TOF/TOF)

Figure 1: Summary of research work plan along with its associated methods

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Gut microbiota and Health

Gut microbiota is the microbial collection of bacteria, archaea and eukarya that have colonized in gut environment and during the evolutionary course of time emerged in a symbiotic relationship with its host (Neish, 2009).

The presence of gut microbiota is indispensable for the ultimate fitness of host in terms its immunity, metabolism and mental health. Though it is genetically determined but to larger extent it is determined by environmental factors including diet and drug in particular. Extensive studies based on both human and animal models have justified the relation between gut microflora and health (De Palma *et al.*, 2017; Levy *et al.*, 2017; Rothschild *et al.*, 2018). Latest DNA based technologies are used to elucidate the microbial composition of gut and infer their possible functions. In addition to this metabolomics is key to measure various metabolites of intestinal microbiota (Vogtmann *et al.*, 2016; Zhao *et al.*, 2017). Short chain fatty acids and gases are the two main fermented products of microbial metabolism which provide energy and regulate intestinal homoeostasis and destroy cancerous cells (Byndloss *et al.*, 2017). The two indicator of healthy gut are richness and diversity which are negatively correlated with dysbiosis (Cotillard *et al.*, 2013). Remodeling of gut microbiota is the therapeutic approach for prevention and treatment of a variety of psychological and pathological malignancies (Van *et al.*, 2013). The microbial communities of gut influences all body systems through several pathways involving neuronal, enteroendocrine cells, immune cells and its metabolites (Schroeder & Bäckhed, 2016). Gut microbiota is involved in the synthesis of essential vitamins which the host is incapable to produce (LeBlanc *et al.*, 2013). Lactic acid bacteria is very crucial in the production of vitamin B-12 as all the organisms including humans, animals and plants cant synthesize it themselves (Martens *et al.*, 2002). Gut bacteria metabolize bile acids and prevent their conversion to secondary bile acids (Staley *et al.*,2017). All these factors contribute to overall host health as any aberration in these microbiota leads to various metabolic maladies such as obesity and type -2 diabetes (Palau-Rodriguez *et al.*, 2015). Gut microflora has a pivotal role in development of intestinal and systemic immune system specially in the stimulation of CD4 T cells. The germ free cells lack many components of immune system which is restored by their treatment with microbiota (Mazmanian *et al.*, 2005). Microbiota produce many effector molecular

which are recognized by various receptors such as Toll like and Nod like present on epithelial cells generating chemical pathways that lead to strength immune system, improve inflammatory problems and differentiate pathogens from beneficial microbes (Hevia *et al.*, 2015).

Probiotics can noticeably protect mice against food borne infections caused by *Listeria monocytogenes* and *Salmonella typhimurium* (Corr *et al.*, 2007).

- Administration of *L. reuteri* as a probiotic to infants prevent acute abdominal pain, constipation and acid reflux in infants (Indrio *et al.*, 2014; Sung *et al.*, 2013)
- Probiotics are also involved in the regulation of other gastric infections like necrotizing enterocolitis, ulcerative colitis (inflammatory bowel diseases), Crohn's disease and also a few that are induced due to Helicobacter pylori (Isolauri *et al.*, 2000).
- Lactose intolerant individuals develop calcium deficiency in them which can be overcome by use of probiotics which is taken in form of yogurt containing combination of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.
- *Streptococcus spp*., are involved in tooth decay of children having ages between 3-4 years, probiotics lower down the incidence of clinical dental cries (Näse *et al.*, 2001). , LGG probiotics are found to possess antimicrobial potential against *Streptococcus* spp. which is causative agent of dental caries (Silva *et al.*, 1987).
- It is well documented that probiotics is effective for prevention of allergy. Samples from 27 breast-fed infants which were suffering from atopic eczema were treated with hydrolyzed whey formulas added with probiotics. The outcome of the hydrolyzed whey formulas added with probiotics was evalued (*B. lactis* Bb12 and *L. rhamnosus* GG) in 27 breast-fed infants that underwent from atopic eczema.This treatment which continued for sixty days helped to lessen clinical signs and symptoms of atopic eczema (Isolauri *et al.*, 2000). Strains such as *Lactobacillus, Bifidobacteria* and *E. coli* strain Nissle 1917 are found to be anti-mutagenic *in vitro* which might relate to their ability to produce antimicrobials and mutagenic compound. Cytoplasmic fractions of *L. casei* YIT9029 and *B. longum* HY8001 was demonstrated to repress the reproduction of tumor cells when they were supplied orally to mice as nutritive additive (Lee *et al.*, 2004)
- **2.1.1 Probiotics use in human nutrition**

Elie Metchnikoff proposed that longevity of Bulgarian population was due to the intake of fermented food such as dahi which later gave genesis to the idea of probiotics. Friendly healthy bacteria displacing the pathogenic ones became the scientific rationale of that healthy life (Ringel *et al.*, 2012). Henry Tissier compared the stool of diarrheal patients versus healthy individuals and found difference in their microbiota (Tissier, 1907).

Probiotics are applied in different products such as feed supplements, fruit juices, chocolates, even in meat products. Dairy products are the mostly readily available probiotic sources. Extensive range of commercially available probiotics are used by European population e.g. (bifisoft, bifidus, bifit), fermented milk with low viscosity (cultured buttermilk, yoghurt drink) and non-fermented products(ice cream, vivi vivo) (Tamime *et al.*, 2005). Probiotics resists a variety of gastrointestinal tract infections by keeping the microbiota composition in balance (Liong, 2007).

2.2 Probiotics (Definition, role and selection)

Probiotic is derivative from the Greek word "pro bios" means for life (Soccol *et al.*, 2010). In 1965 Lilly and Stillwell were the first to use the term probiotics they defined it as: "Influences secreted by the microorganisms that encourages the growth of the other microorganisms". Probiotic definition kept on modifications by later on scientists. In 1974 Parker gave the concept of probiotics as: substances or microbes that have the ability to restore microbial balance. According to (Salminen *et al.*, 1998) probiotics are food comprising of live microorganisms that are advantageous for health".World health organization defines probiotics as "live microorganisms either used as single strain or mixture of strains that provide health benefits to its consumer when taken in recommended amounts" (Rehaiem *et al.*, 2014). Probiotics areee defined as the microorganisms that are beneficial to its host (Arora & Baldi, 2017). Probiotics improve physiology of host and also acts as a substitute for antibiotic use (Banerjee *et al.*, 2017). The microorganisms either in viable, dead and their components rendering health benefits to its host when used for specified period of time at a specified concentration are termed as probiotics (Plaza-Diaz *et al.*, 2019).The functional foods mostly contain lactic acid bacteria which exhibit probiotic properties. Probiotics helps in the prevention of various digestive disorders (Plaza-Diaz *et al.*, 2019).The most commonly used probiotics belong to Lactic acid bacteria specifically *Lactococcus, Pediococcus, Enterococcus, Bifidobacteria* and yeast such as *Saccharomyces boulardii* (Kerry *et al.*,

2018). Due to unique morphological, physiological and metabolic characteristics lactic acid bacteria makes them highly effective probiotics. These beneficial microbes produce a variety of health promoting organic acid and aromatic compounds. Antimicrobial peptides secreted by probiotics is lethal to pathogens without compromising host health (Siripornadulsil *et al.*, 2014). Probiotic function is determined by the host, microorganism and insertion site. Probiotics are efficiently tolerant to GI tract stress barriers such as acidic secretions, pH, enzyme and bile acids. Probiotics ferment carbohydrates to short chain fatty acids which lowers down celiac pH. SCFA are main source of energy for the gut cells and have a key role in the metabolism and physiology of the gastrointestinal tract. The proliferation of cells of the gut, programmed cell death, production of mucin, metabolism of lipid, detoxification of compounds, and competitive exclusion of pathogens and immune stimulation seems to be intermediated by SCFAs (Chapman *et al.*, 2011; Levy *et al.*, 2017; Shapiro *et al.*, 2014).

2.2.1 Selection of probiotics

The probiotic activity is strain specific(Azaïs-Braesco *et al.*, 2010). High throughput molecular techniques are required for accurate identification of the strain. Most of the probiotics available are considered to safe and ensure health benefits to its consumer. Extra precautionary measures should be advisable while selecting and monitoring probiotics for immunocomprised patients as the outcomes could be sepsis, fungemia and gastrointestinal ischemia. Therefore evaluation of risk benefit ratio of probiotics should be thoroughly assessed while prescribing for patients (McClave *et al.*, 2009).

2.2.2 Adherence to the epithelial cells

Probiotics should adhere to epithelial cells for their survival and subsequent colonization (Harzallah & Belhadj, 2013). Numerous components of bacteria such as fimbrial cell matrix material of bacterial cell, mucous like proteinaceous adhesions, lipotehoic acids, S-layer proteins and cell surface hydrophobicity support this adhesion. (Granato *et al.*, 1999). Epithelial cells secrete mucin which is chemically glycoprotein. Mucin being subject to periodic dissociation by proteases, probiotics should get accession to epithelium lest by thrown away by detached mucin (Smet *et al.*, 1995). Surface proteins of bacterial cells attach either directly with epithelial layer or via extracellular matrix. This extracellular matrix is made up of mixture of proteins

secreted by epithelial cells. These proteins in the case of mammalian epithelial cells are usually laminin, collagen, and fibronectin (Kapczynski *et al.*, 2000). Many fibronectin proteins (a dimeric glycoprotein) in the cell surface of *Streptococcus* species are found to be involved in adhesion to the epithelial cells for example FbpA. FbpA is a surface associated protein found in *S. gordonii* (Christie *et al.*, 2002). Its importance can be assessed from this experimental fact that the removal of FbpA from the bacterial cell surface causes 76% decrease in the adherence capacity to the epithelial cells (Buck *et al.*, 2005).

2.2.3 Bile salt Tolerance

Bile is aqueous yellowish green aqueous mixture chemically composed of bile acids, phospholipids, and cholesterol and biliverdin pigment. It helps in emulsification and digestion of lipids. It is synthesized from cholesterol in liver and after ingestion it is released in small intestine through gall bladder in which it is stored (Hofmann & Roda, 1984).

Prior to secretion in small intestine glycoconjugation and tauroconjugation of bile occurs.These also causes to dissolve the bacterial membrane thus having the antimicrobial activity, thus the probiotic strains must have the ability to tolerate bile concentration (Begley *et al.*, 2006). Colonic microorganisms chemically change the conjugated bile by deconjugation, dehydrogenation, dehydroxylation and deglucoronidation (Taranto *et al.*, 1998). Bile salt hydrolase enzymes, responsible for the deconjugation, are found in many microorganisms and goes fit to the choloylglycine hydrolase family of enzymes. They catalyzes the process of hydrolysis of tauroconjugated and glycoconjugated bile acids in to their respective deconjugated forms and amino acids. These enzymes are intracellular and are insensitive to oxygen. Their pH is slightly acidic that is approximately between 5 and 6 (Grill *et al.*, 2000). Deconjugation by these enzymes cause the release of amino acids which serves as a source of nitrogen, carbon and energy as the taurine is broken down to sulphate, glycine aminoacid is converted to CO_2 and NH_3 (Van Eldere *et al.*, 1996). Presence of bacteriolytic enzymes like lysozyme and antimicrobial peptides might destroy probiotics which are safeguarded by them by the protective role of bile salt hydrolases which change the fluidity, charge and tensile strength of probiotic cell membrane making them least prone to damage by these antimicrobials peptides (Peschel, 2002) (Peschel, 2002; Taranto *et al.*, 1998). Conjugated forms of bile salts are highly toxic

they can damage the cells by increasing acidity, probiotics having bile salt hydrolase activity survive by deconjugating these bile salts (Corcoran *et al.*, 2005).

Different genera of gut microbiota including *Lactobacillus* (Christiaens *et al.*, 1992; Lundeen, 1990), *Bifidobacterium* (Grill *et al.*, 2000), *Bacteroides* (Kawamoto *et al.*, 1989) *Clostridium* (Gopal *et al.*, 1996) and *Enterococcus* hydrolyze the bile salts (Franz *et al.*, 2001). Evaluation of bile tolerance of probiotics can be achieved by incubating them in milk-yeast medium having bile added in different concentrations followed by monitoring of viable cell count and pH of media (Goktepe, 2006). It is supposed that toxic nature of conjugated bile is due to its acidification causing nature same as organic acids. It has also been proposed that the BSH enzymes are detergent shock protein (Adamowicz *et al.*, 1991).

2.2.4 Competitive exclusion (CE)

The competition between two species for ecological niche in which one that better adapted will replace the inferior is basis for competitive exclusion (Vine *et al.*, 2004). Probiotics adhere to the intestinal mucosa can prevent the attachment of pathogens (Benno, 1992; Vine *et al.*, 2004). A study based on urinary infection in rat model demonstrated that *Lactobacillus* competitively excluded *Enterococcus faecalis* (Velraeds *et al.*, 1996). It was observed that the heat-killed *L.acidophilis* strain LB which was connected to Caco-2 line disallowed the diarrheagenic *E. coli to* adhere there and this preventive action of *L. acidophilus* was positively correlated with its concentration (Chauvière *et al.*, 1992). It was observed that the strains such as *bifidum M6 and B. bifidum* primarily stopped certain enterero pathogens such as *Clostridium difficile* ATCC 9689, *Enterobacter sakazakii* ATCC 29544, *Salmonella enterica* serovar *typhimurium* ATCC 29631, *Escherichia coli* NCTC 8603 and *Listeria monocytogenes* ATCC 15313 (Gueimonde *et al.*, 2007). The range of expulsion was strain specific and was found to be 15-70%. *Bifidobacterium* having high affinity for receptors present in mucous might be responsible for the expulsion of pathogens.

2.2.5 Co-aggregation of probiotics

Coaggregation, autoaggregation and displacement of pathogens by probiotics is found to be an impotant tool to remove pathogens from digestive tract. Symbiotic interaction of probiotics with commensals is vital for their survival and long term sustainability in their host. It has been conjectured that mixtures of probiotic bacterial strains work well

as compared to single strain probiotic (Collado *et al.*, 2007). Coaggregation of probiotics functions a physical and chemical barrier that prevent the pathogens from establishing there (Collado *et al.*, 2007). *Lactobacilli* have been reported to inhibit uropathogenic bacteria by co-aggregating with some uropathogenic bacteria thus inhibiting their growth (Redondo-Lopez *et al.*, 1990). Co-aggregation of *Lactobacillus acidophilus, Lactobacillus gasseri,* and *Lactobacillus jensenii* with few pathogens like *Candida albicans, E. coli,* and *Gardnerella vaginalis* was observed by (Boris *et al.*, 1998).

2.2.6 Antibiotic resistance in probiotics

Consumption of probiotics is parallel with its safety assurance. Those strains which are susceptible to antibiotics are generally considered as potential probiotic candidate (Hummel *et al.*, 2007). Particular strains of Lactic acid bacteria are carriers of transmissible antibiotic genes. Such strains can transfer resistant genes to pathogenic bacteria of the gut. These resistant strains can be passed on from animals to humans through food chains (Singer *et al.*, 2003). European Food Safety Authority

evaluate completely the antibiotic resistance pattern of any strain prior to its claim as probiotics status (Authority, 2008). It is very important to understand that the resistance is either intrinsic or acquired by chromosomal mutation or horizontal gene transfer. Lactic acid bacteria are sensitive to the antibiotics that target cell wall such as penicillin and β-lactamase but they are resistant towards the cephalosporin. They also show high resistance against vancomycin. Most of the lactic acid bacteria are less sensitive to the nucleic acid inhibitors. Lactic acid bacteria are also sensitive to the low concentrations of protein synthesis inhibitors like chloramphenicol, macrolides, lincosamides and tetracycline but they show high resistance towards the aminoglycosides (Gueimonde *et al.*, 2013). Most of the bacteria present in this genus are resistant to vancomycin. Mechanism of resistance to vancomycin is well known. Vancomycin interacts with the peptidoglycan precursors of the bacterial cell wall and makes a bond with the D-alanine/D-alanine boundary of the pentapeptide that results in the inhibition of the process of polymerization of the peptidoglycan precursors. Many species of lactic acid bacteria have developed the resistance against the vancomycin by replacing the D-alanine/D-alanine terminal residues by the D-lactate or D-serine in the muramylpentapeptide that results in the prevention of vancomycin bond formation. Binding of the erythromycin to the ribosomes has reduced due to the single known

mutation in 23S rRNA gene. This resistance cannot be transfer to the others (Singer *et al.*, 2003).

L. acidophilus, L. delbrueckii subsp. Bulgaricus, L. johnsonii, L. reuteri and L. plantarum have been reported for the presence chloramphenicol resistance genes that is cat. This genes is reported to be present on the plasmid that is a mobile element so there are the chances of antibiotic resistance gene to be spread to the other microbes or even to the pathogens by the process of conjugation but in *Bacillus* species this resistance gene cat (Bcl) is present on the chromosome so they are safe to be used as probiotics (Mayrhofer *et al.*, 2011). Vat (E), a resistance gene for Quinupristin– dalfopristin, is present on the chromosome in *Lactobacillus* species and are thus safe. Tet(W), tet(M), tet(S), tet(O), tet(Q), tet(36),tet(Z), tet (O/W/32/O/W/O), tet(W/O) and efflux pumps tet(K) and tet(L) are the resistance genes to be present in lactobacillus species of bacteria for tetracycline resistance. These genes are also reported be located not only on the chromosomes but also on the plasmids and transposons (Gueimonde *et al.*, 2013). It is concluded from the above that most of the microbes that are present in our food having a massive pool antibiotic resistance genes and their consumption can influence their presence, existence and dynamics in the body of the host (Gueimonde *et al.*, 2013). Those probiotics should be selected which do not carry antibiotic resistant genes. The unstrained use of antimicrobial agents has aggravated the spread of resistant microbes. Therefore stringent check and balance is needed while using such drugs.

2.2.7 Acid tolerance

Most of the lactobacilli are resilient to low pH. When the pH value of the cellular matrix reaches to a threshold value the cellular function stop cell becomes dead. F0F1-ATPase is present in Gram positive bacteria which has the capability to elevate the internal pH of cell even when the extracellular pH is low. It activates at low pH and its regulation occurs at transcriptional level. F0F1-ATPase enzyme composed of multiple subunits, F0 integral membrane portion, F1 catalytic portion consists of α, β, γ, δ and ε subunits that is involved in the hydrolysis of ATP. F0 is composed of a, b, c subunits which is involved in the translocation of protons.Several mechanism adopted by cells to regulate the low pH resistance included changing metabolic pathways,cell membrane composition alterations, proton pumps, neutralization. Latest biotechnological approaches are also used to resist low pH (Liu *et al.*, 2015; Wang *et al.*, 2018).

2.2.8 Antibiotic production by probiotics

The property of antimicrobial production by probiotics is applied to cure intestinal infections. Various antimicrobials are reported such as fatty acids, organic acids, diacetyl acetoin peroxide and extensively studied "bacteriocins" (Simova *et al.*, 2009). Antimicrobials are divided into two classes namely low molecular mass having molecular mass less than 100DA and high molecular mass molecules such as bacteriocin having molecular mass less than 1000DA (Tejero-Sariñena *et al.*, 2012).

Table 2. 1: Types and classification of antimicrobials produced by probiotics microorganisms (Gueimonde* et al.*, 2013).

Category	Classes	Examples
High molecular mass(Bacteriocins)	ClassI (Lantibiotics lanthione and β-lanthionine)	lactocin S, lacticin 481 and nicin Mersacidin Actagardin Mutacin II
	Class II (Non lanthione heat stable molecular mass <10 kDa	Plantaricin JK Plantaricin EF enterocin B acidocin B and enterocin P
	Class III (bacteriolysins molecular mass 30kDa	Enterolysin A
	Class IV	Lactacin
Low molecular mass	Fermentation's metabolites of bacteria	Lactic acid, ethanol, H_2O_2, CO_2

2.3 Fish Gut Microbiology

Gut is readily colonized just after hatching under the influence of water communities which is later on further added when the feed intake by fish starts.

Gastrointestinal microflora is crucial for the survival and growth of the host. Microbial ecology of fishes is studied to limited extent as compared to terrestrial organisms as it is a newly emerging field.

The gut microbiome is crucial for survival of fish by participating in nutrient metabolism, immune modulation energy homeostasis and preventing from infectious (Costello *et al.*, 2009; Merrifield & Carnevali, 2014). Extensive understanding of fish gut microbiota has a key importance in aquaculture (Austin, 2006; Grice *et al.*, 2009). The major intestinal microflora of fishes include *Proteobacteria, Actinobacteria,*

Bacteroidetes, Firmicutes and Fusobacteria (Gómez & Balcázar, 2008). Bacterial species that are commonly present are *Bacillus*, *Vibrio*, *Micrococcus*, *Clostridium*, *Bacteroides* and *Pseudomonas* genera, as well as various species of lactic acid bacteria (LAB) (i.e.*Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Pediococcus*) (Romero *et al.*, 2014). Yeast, protozoa and viruses are commonly present communities of fish gut. (Merrifield & Rodiles, 2015). The fish microbiome change with respect to the life stage of fish (Bakke *et al.*, 2015; Burns *et al.*, 2015). Gut microbiology is intensely investigated by using both culture dependent and culture independent techniques.

2.4 Techniques to study fish gut microbiology

2.4.1 Culture Dependent Te chnique (Bakke *et al.*, 2015)

Culture dependent technique though economical is able to reveal only 30% of the total microbial diversity (Eckburg *et al.*, 2005; Guarner & Malagelada, 2003; Nandi *et al.*, 2017).Certain microorganisms cannot be cultured because of the deficiency of complete knowledge regarding their growth requirements. With the advent of techniques like chips and micro droplets it is now possible to cultivate previously uncultivable microbiota (Ingham *et al.*, 2007).

2.4.2 Culture independent techniques

State of the art molecular techniques such as PCR cloning and DNA sequencing, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) has enable us to the understand complex gut microbial communities (Li *et al.*, 2012; Roeselers *et al.*, 2011) Though highly sophisticated but most of these techniques are able to identify dominant microorganisms leaving majority of diversity yet to be explored (Cardenas & Tiedje, 2008). Advanced sequencing has made it easy to analyze microorganisms. Large number of microbial samples can be processed with the aid of sequence tags and it is cost effective (Brady *et al.*, 2000). These techniques are also being applied for analyzing gut microbial flora of humans as well as fish (Sokol *et al.*, 2008; Vrieze *et al.*, 2010). Wide variety of approaches collectively known as “metagenomics” are also in practice to explore the microbial communities without culturing them (Venter *et al.*, 2004). This approach investigates three main interconnected levels (sample processing, genome sequencing and functional analysis) that finally discover microbial community functioning (Venter *et al.*, 2004). Most often used technologies for the analysis of fish gut microbiome are

Pyrosequencing and Illumina technologies. Earlier 454 GS FLX Titanium was used to study hindgut communities of Atlantic salmon for 13 months in different life phases and seasons in relation to changes commercial diets. He observed that dynamics of hindgut communities has no significant relation with changing commercial diet (Zarkasi *et al.*, 2014). In a prevous study the effect of feed composition and its abundance on the function and composition of gut microflora in grass carp using illumina Hi-Seq 2000 was analyzed (Ni *et al.*, 2014).

2.5 Factors influencing gut microbiology

Fish gut microbiotia is prone to be influenced by external environment. A multitude of factors like feed associated microbes, pH, temperature, salinity, precipitation, particular matter shape the gut architechture of fishes and well as surrowding environment (Austin, 2006; Cahill, 1990).

2.5.1 Environmental factors

It was reported that the variation in surrounding water temperature affect the composition of gut microbial community of *Senegese sole Solea senegalensis* especially a change in the percentage of vibrio was observed when studied by culture dependent method (Martin-Antonio *et al.*, 2007). The influence of high precipitation is correlated with abundance of *Salmonella* in the GIT of warmouth *Lepomis gulosus* (Mendoza II, 2011). The variation in gut microbiota was found to be correlated with season and geographical location (Le Nguyen *et al.*, 2008).

2.5.2 Age and size of fish

The composition of microbial communities is correlated with the age of the fish . Prior to onset of first feeding gut microorganisms from surrounding water begins to colonise in fish gut. The change and diversification in fish gut flora is observed post larva feeding (Romero & Navarrete, 2006). This complexity and shift in microbiology linked with food associated microbles or gut adaptation in new microbial environment (Brunvold *et al.*, 2007). It is suggested that permanent fish gut microflora is shaped from egg microbiota and surrounding water rather than food taken by fish (McIntosh *et al.*, 2008). Though fishes acquire rather stable microflora by 50th day of its hatching through food or mucous but such microbial niche vary constany with age (Dimitroglou *et al.*, 2011).

2.5.3 Diet type and composition of gut

Disease prevention and enhanced productivity in fishes is linked to food supplied to it showing its pronounced effects on fish gut communities. Both allochthonous and autochthonous microbiota is influenced by diet given to fishes (He *et al.*, 2011) but some studies negate the effect of diet on the composition of allochthonous microflora (Silva *et al.*, 2011). Most commonly changed genera are *Pseudomonas*, *Vibrio* and *Carnobacterium* (Dimitroglou *et al.*, 2011; He *et al.*, 2011; Ringø *et al.*, 2008; Ringø *et al.*, 2006).

2.5.4 Fish species

Species specificity influence on gut microbial community composition has been reported in many past studies. The type of host (fish species) significantly influence the internal gut microbiota, however mechanism by which host select the microbes is not fully understood. It was reported that zebra fish collected from wild and other rearing conditions shared similar microbial communities despite of variation in environmental conditions (Roeselers *et al.*, 2011). In another study (Smriga *et al.*, 2010) observed significant shift in allochthonous microbial load in surgeonfish *Acanthurus nigricans,* two-spot red snapper *Lutjanus bohar and* parrotfish *Chlorurus sordidus* sampled from same coral reef. These differences might be either diet or specie specific It was reported that digestive tract of the Sea trout and Atlantic salmon harbored distinguishable intestinal microbiome at genera and species level (Skrodenytė-Arbačiauskienė *et al.*, 2008).

2.6 Aquaculture and probiotics

2.6.1 Aquaculture an introduction: Feeding a growing population

Sea food has been an indispensable high quailty protein sources since old times. Mismanagement of wild capturing practives has drastically limited their productivity showing its inability to satiate the hunger of rising world population which is predicted to be 9.3 - 9.6 billion by the year 2050 (Ezeh *et al.*, 2012; FAO, 2014).Aquaculure is an approach dealing with both protein demand and aiming to lessen pressure on wild resources. Food and agriculture organization stated that aquaculture had generated a revenew of US$ 130 billion in 2012 (Bostock *et al.*, 2010; Defoirdt *et al.*, 2011). It is the need of hour to establish the sustainable aquaculture. Enormous increase has been observed in the production of farmed fish and shellfish during last decade (Naylor *et al.*, 2000) the time scale from 1960-2014 has shown a sharp increase in fish

consumption worldwide reaching a level of 87% or more than 146 million tons. It is anticipated that global demand for fish based food will cross more than 2 million tons by commencement of 2020 (FAO, 2006).

The most profitable species used for aquaculture are African catfish (*Clarias garipienis*), turbot (*Psetta maxima*), tilapia (*Oreochromis niloticus*), cod (*Gadus morhua*) and tuna (*Thunnus* spp.)(Nieuwegiessen, 2009).

2.6.2 Economic importance of aquaculture: Global and Pakistan

Health and income generation are the main outcomes associated with fisheries and aquaculture. It has generated employments throughout the world thus providing opportunity to earn. Fish stays to be one of the broadly merchandised food product at the global scale, the main contributors being developing countries. Fishery activities is a source of gross domesticated product and it generates government revenues through fishery agreements and taxes. According to FAO statistical analysis held in 2014, employees in fishery sector are classified as full time fishers and part-time processors. African people of age's range 15-64 were associated with this sector which represent 21% of their total population. The most recent estimates indicate that 58.3 million people were engaged in the primary sector of capture fisheries and aquaculture in 2012. Of these, 37 percent were engaged full time, 23 percent part time, and the remainder were either occasional fishers or of unspecified status. In 2012, 84 percent of all people employed in the fisheries and aquaculture sector were in Asia, followed by Africa (more than 10 percent), and Latin America and the Caribbean (3.9 percent). About 18.9 million (more than 32 percent of all people employed in the sector) were engaged in fish farming, concentrated primarily in Asia (more than 96 percent), followed by Africa (1.6 percent), and Latin America and the Caribbean (1.4 percent) (!!! INVALID CITATION !!!). An estimated 56.6 million people were engaged in the primary sector of capture fisheries and aquaculture in 2014, of whom 36% were engaged full time, 23% part time, and the remainder were either occasional fishers or of unspecified status (FAO, 2016). According to the FAO annual reports though aquaculture is flourishing globally but, in Pakistan it is still an emerging sector, contributing 1% to country's economy. Fresh water carping is the mainstream aquaculture product in Punjab, Sindh and Khyber Pakhtoon Khawa Province [KPK]. The colder areas of Pakistan exhibits immense potential for trout growth but it is produced at a small scale.Progressive

approaches are necessary for the development of aquaculture in future as Pakistan has got immense potential for it in near future.

2.6.3 Major challenges of aquaculture

One of the bottleneck associated with with farming is prevelance of infectious and non-infectious diseases which can be prevented by aqua feed and disease prevention. (Dobsikova *et al.*, 2012). Preventive approach targeted bio-based solutions are far more better option for growers than customary chemicals and antibiotic based remdial measure, considering the issues such as antibiotic resistance,environmental damage and drug residues. Scientists are developing feed based additives such as probiotics as ecofriendly approach to save this million dollar industry worldwide (FAO, 2002; Li & Gatlin III, 2005; Ringø *et al.*, 2010).

2.6.4 Pacing with rise in demand along with sustainable approach

Fisheries are one the fastest food producing sector and richest source of animal protein. According to an estimate at global scale more than 25% demand for animal protein is satisfied by fishes and it is on increasing trend with the passage of time (Naylor *et al.*, 2000). As the industry has grown multifold tremendous development is seen in its relevant sectors such as commercial diets, growth promoters, antibiotics, and several other additives (Wang *et al.*, 2008). However, economic losses are the one of the highlighting challenge of modern aquaculture.Chemotherapeutics not only kills pathogens but it is devastating the environmental sustainibilty. Human health is also at stake due to spread of resistant strains and drug residues by these agents (Radu *et al.*, 2003). The desired outputs such as better food productivity, healthy environment and economic benefits can be achieved by adopting alternate approaches (Díaz-Rosales *et al.*, 2006).

Probiotics and prebiotics are used routinely in aquaculture to promote fish growth, prevent diseases and stimulate immunity in fishes (Irianto & Austin, 2002a). Several commercially available probiotics are either added in rearing water or given as feed additives feed additives has reduced the mortality losses in aquatic organisms (Gatesoupe, 1999; Verschuere *et al.*, 2000). Detailed investigation regarding the mode of action of probiotics and effective administration is extremely important for the safe and effective use of probiotics. Partial knowledge regarding used probiotics can lead to metabolic disorder by modulation of microbiota, disturbed immune system,

transmission of antibiotic resistance from probiotics to pathogens (Boyle *et al.*, 2006). An ideal probiotic should be able to inhabit, establish and proliferate in host gut (Flegel & Pasharawipas, 1998).

Probiotics are strain specific in their action on the host, different strains of same species act differently on the host, sometimes imparting opposite effects (Aureli *et al.*, 2011). *Shewanella putrefaciens* and *Shewanella baltica* both used as probiotics differently trigger respiratory burst activity (Díaz-Rosales *et al.*, 2009).

The strategy to transfer microflora from mature fishes to immature ones had very successful outcomes (Gomez-Gil *et al.*, 2000). Autochthonous bacteria isolated from fish itself or its natural aquaculture are the best choice as fish probiotic (Verschuere *et al.*, 2000). Commercial probiotics are sometimes ineffective which might be due to the fact they don't have fish origin and thus unable to survive and colonize in fish digestive tract (Abraham *et al.*, 2008). Several studies are proposed to study the impact of combination of probiotics blend or probiotic prebiotic (Patterson & Burkholder, 2003). The multispecies contribute to complementary mode of action hence providing increased protection thus contributing animal welfare (Timmermans, 1987). The autochthonous probiotics have better chance of competing with resident commensals and take short time to establish in GI tract and sustain their for some time even after the cessation of their supply (Carnevali *et al.*, 2004).

Combination of probiotics are very efficient to induce local gut immunity (Salinas *et al.*, 2008). Though non spore and spore former both are used as probiotic but the latter are preffered as they resilient to harsh environmental conditions, powerful immunostimulants, having antimicrobial potential and prolonged shelf life (Moriarty, 2003). *Bacillus* spores are used as humans and animals probiotics due to their immuno-stimulatory attributes (Hong & Cutting, 2005).

2.6.5 Dosage of probiotics

The appropriate dose of probiotics is not only necessary for its colonization and stability in its host but also is quintessential for desired health outcomes such as growth, immune stimulation and host protection. According to an estimate of FAO the least concentration required for probiotic food supplement should be 10^6–10^7live probiotic bacteria per gram or milliliter (Bajagai *et al.*, 2016). It was documented that the percentage mortality in *O. mykiss* increased when they were provided with high dose of *L. rhamnosus* (10^{12} CFU g feed-1) as compared to lower dose (10^9 CFU g feed-1)

(Nikoskelainen *et al.*, 2001). it was observed that the immunity in fish is dependent on the concentration of the probiotic which usually vary between 10^{6-8}CFU/g but the dose concentration is variable with respect to host and immunological parameter studies (Panigrahi *et al.*, 2004).

There are various mode of probiotic delivery in aquaculture including bath immersion, dietary supplementation and suspension. Proper colonization of probiotics in gut can be achieved by dietary supplementation. The direct application of probiotics in rearing water may help to keep the its chemical parametrs and biological parameters to an optimum level (Boyd & Massaut, 1999; Zhou *et al.*, 2010).

2.6.6 Environmental conditions

As fishes are poikilothermic organisms the intestine of the fishes acquire the same temperature as of the surrounding water so the probiotics whose optimum temperature is similar as that of water can better survive and impart its benefits to host (Panigrahi *et al.*, 2007). There are certain water parameters namely water quality, hardness, pH, temperature, dissolved oxygen, osmotic pressure and mechanical friction which effect the establishment of probiotics in digestive tract and subsequently their actions on host (Das *et al.*, 2008). High stock density can cause stress in fishes due to which probiotics can't perform efficiently in the host. If the stress is due to salinity or high temperature then probiotics can alleviate this stress (Asli *et al.*, 2007; Taoka *et al.*, 2006).

2.6.7 Duration of treatment

Probiotics showed their impact on host in a duration dependent manner which affects their survival, propagation and activities on the host. The beneficial effects of probiotics on host mostly were observed within 10 weeks of dietary intake of probiotics as manifested by many studies (Nayak, 2010). The time period required to stimulate immune system varies with respect to probiotic strains and also it depends on the parameter of the immune system under study (Choi & Yoon, 2008). Previously conducted study showed that the respiratory burst activity in trout was not noticed during 30 days of probiotic delivery but the same strain caused the induction of respiratory burst activity when fed for 60 days period (Choi & Yoon, 2008). Short term probiotic feeding in many cases results in a sharp decrease in piscine immune reaction which might be due to the failure of probiotic establishment hence no benefits (Panigrahi *et al.*, 2005b)

2.6.8 Probiotic viability and survival

One of the main concern in commercial probiotic production is to maintain sufficient amount of viable probiotics during processing and storage (Gatesoupe, 1999). Refrigeration is foremost requirement for the storage and transportation of probiotic in liquid/frozen form used in aquaculture adding expenditures and inopportuneness for its extensive use in aquaculture. Long shelf life of probiotic and their viability should be assured for commercial probiotic products. Molecular techniques provide accurate identification of probiotics strains for the quality assurance and safety (Wang *et al.*, 2008).

2.6.9 Probiotics and fish immunity

Probiotics modulate immuno hematological parameters to ensure enhanced growth and disease protection (Nayak, 2010). Fish immune system is divisible into innate and acquired immune system. Innate immunity is further divided into humoral immunity in which body fluids participate in immunization (cell free bodily fluid or serum) and cellular immunity in which cells participate in conferring immunity. Highly specialized cells and processes namely B and T lymphocytes are the major component of immune system (Alberts *et al.*, 2002; Janeway *et al.*, 1996).

Functions of vertebrate innate immune system are:1) Employment of immune cells to site of infection 2) activation of complement cascade identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes 3) the identification and elimination of foreign substances from host body tissues by white blood cells 4) triggering of adaptive immune system through antigen presentation 5) performing as a physical and chemical barrier to the infectious agents (Alberts *et al.*, 2002; Janeway *et al.*, 1996).

Lysozyme is an essential tool of innate immune system possessing enormous bactericidal activity (Lindsay, 1986). Numerous studies are conducted regarding role of probiotics affecting the level of lysozyme some studies confirming that they affect (Balcázar *et al.*, 2006; Kim & Austin, 2006; Panigrahi *et al.*, 2004) and some other studies conducted in *O.mykiss* failed to increase the level of lysozyme irrespective of its mode of delivery i-e. Through diet or through water supplementation (Balcázar *et al.*, 2007; Panigrahi *et al.*, 2005b).

Peroxidases which are produced mostly by neutrophils during the oxidative respiratory burst use oxygen radicals to produce hypochlorous acid which kills the infectious

agents (Nayak, 2010). Certain probiotics have successfully reported to elevate the level of peroxidases during oxidative respiratory burst (Brunt *et al.*, 2007; Sharifuzzaman & Austin, 2009) but contradictory studies have also found in literature (Salinas *et al.*, 2008).

Complement system of the fish consist of cascade of different proteins related to degradation and phagocytosis of pathogens by lysis (Janeway *et al.*, 1996). Three biochemical pathways converge to a lytic pathway resulting in direct killing or opsonisation of pathogens. These pathways are a) the classical pathway, b) the alternative pathway; and c) the lectin pathway (Alberts *et al.*, 2002; Holland & Lambris, 2002; Janeway *et al.*, 1996). Classic pathways is dependent on antibodies and it is a part of specific immunity whereas alternate pathway do not require antibodies for activation (Janeway, 2001). Many studies showed that the probiotics supplied either through diet or rearing water elevate the nature complement activity of fish (Panigrahi *et al.*, 2005b; Panigrahi *et al.*, 2007; Salinas *et al.*, 2008). It is an important fact that non-viable probiotics can uplift the natural complement action of fish (Choi & Yoon, 2008).

One of the intrinsic defense mechanism of fishes is respiratory burst activity. Probiotic supplementation via feed or rearing water found to contradictory in their effects, some studies showed that probiotics enhance respiratory burst (Nikoskelainen *et al.*, 2003; Salinas *et al.*, 2005; Salinas *et al.*, 2006; Zhou *et al.*, 2010), while others found that application of probiotics have null effect on respiratory burst activity (Díaz-Rosales *et al.*, 2009; Sharifuzzaman & Austin, 2009).

Stimulated macrophages are demonstrated to produce NO (nitric oxide) which defend the cell by killing viral, bacterial and bacterial infectious agents (Buentello & Gatlin, 1999; Neumann *et al.*, 1995; Tafalla & Novoa, 2000). As the immune systems of fish larvae shirmps and other invertebrates is under developed than aldult and so primarily infection control is mediated by non-specific immune system. (Ogunshe & Olabode, 2009; Verschuere *et al.*, 2000) assessed the capacity of *Lactobacillus fermentum* LbFF4 isolated from Nigerian fermented food (‘fufu’) and *L. plantarum* LbOGI from a beverage ‘Ogi’ to induce immunity in *Clarias gariepinus* (Burchell) against certain selected fish bacterial pathogens.

2.6.10 Probiotics use in fishery

Fish being poikilothermic is under the constant impact of fluctuating surrounding environment. The unexpected alteration of exposed environment in aquatic organisms particularly fishes can suppress both innate and specific immune systems thereby changing biochemical parameters and adversely affects the fish health (Girón-Pérez *et al.*, 2007). Nonspecific immune system plays a major role in protecting fish against infectious agents. Studies based on modification of non-specific immune system are befitting for cure and prevention of fish diseases (Misra *et al.*, 2006b). Infectious outbreaks in aquaculture are controlled by antibiotics, vaccines and chemotherapeutics. Microbial diseases in aquaculture leads to serious economic losses (Acar *et al.*, 2015). The non-selective use of antibiotics is related to the rise in antibiotic resistance which influences the humans through food chain (Nomoto, 2005). There are common transposons and resistant genes between aquatic and terrestrial bacteria which suggests that such transfer between the bacteria of these spheres has been carried through a process horizontal gene transfer between them (Cabello *et al.*, 2013).

Emerging trends in the field of functional feed has been observed in recent time whose concept is based on the provision of diet which impart extra benefits to host rather than only fulfilling nutritional requirement (Li & Gatlin, 2004). Prevention and reduction of disease is preferable than its treatment. Probiotics has emerged as an alternative to customary treatments based on chemicals and antibiotics and is found to be effective in disease control but consideration should to be done while using probiotics in host (Rekiel *et al.*, 2007). Kozka was the first person who introduced probiotics in aquaculture. He got the idea by discovering that probiotics were already in practice in poultry and human diseases. His focus was on increase the growth rate of *Seriola quinqueradiata*, yellow tail by addition of spores of *Bacillus toyoi* in feed (Kozasa, 1986). Bacillus species in 1991 was used to check if it enhanced the productivity of *Penaeus monodon* farming by Porubcan. Improvement of water quality was also done by reducing the concentrations of ammonia and nitrite. *Bacillus* spp. was reported to control and reduce the number of *Vibrio* species in ponds of shrimp, specifically in sediments (Moriarty, 1998). Probiotics in aquaculture are not only anti pathogenic but enhance nutrient digestibility, reproduction in fishes and also improve water quality (Melgar Valdes *et al.*, 2013; Nandi *et al.*, 2017). Application of *Bidifobacterium bifiduim* and *L. acidophilus* reported to protect Nile tilapia completely from *Aeromonas*

hydrophilla (Van Hai, 2015). It was observed that the administration of single or combination of probiotics such as Bacillus and Lactic acid bacteria not only increase the survival but also growth performance in Nile tilapia (Apún-Molina *et al.*, 2009). Extensive studied fish species including *Oncorhynchus mykiss, Seriola dumerili*, and *Sparus aurata* have proven that the probiotics such as *Bacillus, Lactobacillus and Enterococcus* are effective immunostimulant. *Bacillus* and *Lactobacillus* are widely applied in aquacultirng as the confer multiple health benefits including efficient digestion, feed conversion efficiency,increase in weight, antipathogeinc activity specially against vibrio (Afrilasari & Meryandini, 2016; Gao *et al.*, 2017; Yamashita *et al.*, 2017).

2.6.11. Probiotics and improvement of water qualities

The intended use of probiotics in ponds aquaculture is to develop conditions for production purposes. The mechanism of actions to the positive influence on water is partially understood. The fish farmers rely on the elimination of lethal constituents from water to improve water quality in aquaculture. It was found that addition of photosynthetic bacteria was successful in removing a range of toxic and poisonous substances which subsequently improved quality standards of water. The accumulation of compounds such as ammonia, nitrite and nitrate resulted in contamination is a matter of grave concern in aquaculture (Li *et al.*, 2012). The vulnerability of the cultured aquatic species to high concentration of these compounds is generally species-specific, but in high concentrations, these compounds may be extremely detrimental and cause mass mortality in all cases. It was reported that the *Lactobacillus spp.* JK-8 and JK-11 simultaneously removed nitrogen and pathogens from contaminated shrimp farms (Ma *et al.*, 2009). Water quality can be upgraded by adding probiotics particularly *Bacillus* spp. which is evident from preceding studies (Kolndadacha *et al.*, 2011; Verschuere *et al.*, 2000).

2.6.12 Probiotics as growth promoters

Aquaculture is primarily benefitted by probiotics as it promotes growth of the cultured species. The probiotics might manipulate the gut microbial community by changing its composition towards health promoting microflora (Bai *et al.*, 2013). Probiotics have the ability exclude pathogens and improve host growth with no side effects. Earlier it was observed that tilapia fed on probiotics improved their growth (*Oreochromis*

niloticus). He concluded *Micrococcus luteus* as probiotic not only showed high growth rate but also found to maximize feed conversion ratio in the fishes suggesting *M. luteus* to be potential probiotic in fish rearing in aquaculture (Yassir *et al.*, 2002). Lactic acid bacteria reported to promote growth in juvenile carp but were ineffective in Sea bass (Noh *et al.*, 1994). Previously conducted studies have validated that the probiotics are associated with improved feed digestibility, enhanced growth and increase in weight in salmonids (Merrifield *et al.*, 2011). The rain trout fed on mixture of *B. subtilis* and *B. licheniformis* added diet resulted in improved growth parameters in a feeding trial continued for a period of sixty days (Bagheri *et al.*, 2008). It was stated that addition of probiotics in feed had improved feed digestibility due to that fact they supply a lot of enzymes and growth factors that are necessary for digestion and growth (El-Haroun *et al.*, 2006)

2.6.13 Disease prevention

Probiotics or their products are used for the prevention of disease in terrestrial animals, humans and aquaculture. This friendly microbes either inhibit or even able to eliminate potential pathogens.The proposed modes of prevention includes increasing their resistance to disease or production of inhibitory biological elements such as antibiotics, antibacterial substances, siderophores, bacteriolytic enzymes, proteases and protease inhibitor, lactic acid and other organic compounds like bacteriocins, hydrogen peroxide (Sang, 2009) and butyric acid production that prevent the disease onset by combating pathogens (Pan *et al.*, 2008). Probiotics are in intimate attachment with intestinal walls and in close proximity of culture species thus preventing their multiplication (Verschuere *et al.*, 2000). It is not assured that a probiotic working proficiency in *in vitro* conditions warrants the same adeptness in living system also due to interplay of multiple factors in vivo conditions including host response, pathogens and environmental factors which might influence its efficiency (Pandiyan *et al.*, 2013; Riquelme *et al.*, 2000). The antimicrobial property of probiotic *Pseudomonas fluorescens* AH2 persisted even after seven days as recorded as per an in vitro study (Skjermo *et al.*, 2006). It was observed *Streptomyces* spp. produced lymphostin antibiotics which inhibited other disease causing bacterial agents (Miyanaga *et al.*, 2011a). The antibiotic carbapenem was produced from different species of Streptomyces (Otake *et al.*, 1997).

Certain probiotic bacteria were effective against viruses. The marine algae and bacteria were observed to produce certain extracts which were able to inactivate viruses. Balcazar found that the *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp. obtained from salmon hatcheries had antagonistic effects against infectious hematopoietic necrosis virus (IHNV) (Balcázar *et al.*, 2007) .

2.6.14. Contribution of probiotics to digestion serving as nutrient and enzymatic source

Probiotics not only provide extracellular enzymes such as proteases, amylases and lipases but also contribute nutrients such as amino acids, vitamins and fatty acids thus making digestion efficient and henceforth rendering health benefits (Balcázar *et al.*, 2006; Dimitroglou *et al.*, 2011). Enzymes from marine microorganisms are more stable than similar ones obtained form the terrestrial sources: among them variation of environmental conditions exit. The enzymes from marine environment not only promotes digestion but also serves as quorem sensing quinchers thus enhancing disease resistance in their host can be effective probiotics in aquaculture (Nguyen & Nguyen, 2017). *Bacteroides* and *Clostridium* sp isolated from fish were found to be the source of nutrition especially fatty acids and vitamins (Sahu *et al.*, 2008). Certain microorganisms such as *Agrobacterium* sp, *Pseudomonas* sp*., Brevi-bacterium* sp*., Microbacterium* sp*., and Staphylococcus* sp may play their role in nutritional processes of *Salvelinus alpinus* (Ringø *et al*., 1995).

2.6.15. Probiotics role in reproduction

Properly managed aquaculture is high economy generating project. The mainstay of aquaculture production is associated with reproduction capacity which thus determines the financial gain of any undertaken projects. Multiple determinants are involved in the control of reproduction such as diet, environment and species of fishes. Nutrition is utmost important in every phase of reproductive cycle in both male and female. A latest trend in research is inclined towards understanding of probiotic action in reproduction, especially in descendants with distinctive focus on marine species. Positive modification of reproductive process by probiotics is an emerging trend. Probiotics administration promotes a series of changes that include the increase of egg laying capacity (Miccoli *et al.*, 2015), larval survival as well as development of gonads (Avella *et al.*, 2012). It is finally deduced that probiotics has a positive correlation with the

increased reproductive capacity(Carnevali *et al.*, 2013). Probiotics potential to enhance reproductive process by increasing fecundity, larval survival and gonadal development had been studied in zebra fish indicating overall positive impact on reproductive process (Avella *et al.*, 2012; Gioacchini *et al.*, 2010; Miccoli *et al.*, 2015). Similar results were reproduced in in the brackish species *F. heteroclitus* (Lombardo *et al.*, 2011), while sperm quality was found to be enhanced in European eel (Vílchez *et al.*, 2015).

Trials conducted on different fishes sush as zebrafish, brackish species *F. heteroclitus* confirmed this fact (Lombardo *et al.*, 2011), while an improvement of sperm quality was found in European eel (Vílchez *et al.*, 2015). Probiotic bacteria contribute to overall health of the host thus principally is an appropriate choice as feed additive. It was demonstrated that the intestinal isolate, *B. subtilis* from *Cirrhinus mrigala* improved egg laying capacity, viability, gonadal maturity and number of fry when it was in incorporated to diet of ornamental fishes for a period of a year. It was suggested that the vitamins B synthesized by the probiotic, especially vitamin B1 and B12, contribute in lowering the number of deceased or deformed alevins (Ghosh *et al.*, 2007). A positive correlation was found between the egg laying capacity, sexual maturity and production of fingerlings and supplementation of commercial probiotic Primalac. This commercial mixture was comprised of four lactic acid producers (Abasali & Mohamad, 2010).

3. Material and Methods

3. Material and Methods

The present research work was conducted at Microbiology research laboratory in collaboration with fisheries and aquaculture laboratory at the Faculty of Biological Sciences, Quaid-i-Azam University Islamabad-Pakistan. The research work was designed in three phases. First phase was based on invitro screening for probiotics followed by second phase that was application of selected probiotics in fish and its impacts on physiology. Third phase was targeted to the study of fish gut microbiology both by culture dependent and independent methods. The strains used in the present study were from different sources (fermented milk product, silage and fish gut. Moreover, some already isolated strains including *Geotrichum candidum* QAUGC01 (KT280407) and *Enterococcus faecium* QAUEF01 (KP256006) were isolated from indigenous fermented milk product Dahi, *Enterococcus hirae* QAUEH01 (KP256015) from silage. These strains were applied in experiment both in single form and in coculture with *G. candidum* QAUGC01. A commercial probiotic containing mixture of *Lactococcus* sp., *Lactobacillus* sp. and yeast (undeclared strains) was used as positive control. The preliminary identification of lyophilized fish isolates were done on FTIR which were further identified by 16S DNA sequencing.

3.1 Phase-I

Isolation, characterization and screening of strains for probiotic potential

3.1.1. Isolation of microorganisms from gastrointestinal tract of *L. rohita* (Rohu)

The isolation of microbial strains was done from *L. rohita* (rohu) intestinal tract collected from wild stream water and aquaculture center at Quaid-i-Azam University. Samples were processed immediately after collection and dissected aseptically. Gastrointestinal tracts were taken ten grams of each sample was homogenized and diluted up to four folds in sterile normal saline (0.9% NaCl). From each dilution 0.1 ml diluent was poured on prepared MRS agar, Nutrient agar, Oxytetracycline glucose agar (OGA) and Tryptic Soy Agar (TSA) (Thermo Scientific ™ Oxoid ™ UK) plates by spread plate method. The inoculated plates were incubated for 24 hours at 37°C except OGA plates were incubated at 28°C for 48 hours. Discrete colonies were selected for sub- culturing on respective media. Gram positive and catalase negative isolates were further sub cultured and purified on TSA. The selected pure isolates were preserved in 30% Glycerol (Sigma Aldrich, Eastleigh, UK) amended with culture broth, and stored at -80˚C for further use.

3.2 Screening for Probiotic Potential of Microbial Isolates

The *in vitro* screening was done for the all the isolates form different sources. The probiotic potential of isolates was determined by assessment of indicator characteristics including survival under mimic gut condition by testing tolerance against acid and bile salt. The adherence ability, antibiotic sensitivity, and antagonistic ability against pathogens was tested.

3.2.1 Determination of acid tolerance

Acid tolerance was determined by the method devised by (Singhal *et al.*, 2010) with some modifications. Both bacteria and yeast strains were grown in TSB broth at 37°C and 30°C respectively for 24 hours in shaking incubator (Thermoscientific, UK) at 120rpm. The 100ul aliquot of the active cultures were adjusted to pH 2,5,7 and 9 with 1 N HCl and were incubated for 2 hours and bacterial growth was monitored by measuring absorbance with spectrophotometer at 600 nm. All the experiments were performed in triplicate. For the combination of yeast and bacteria equal volume of both were mixed and optical density were calculated afterwards .

3.2.2 Determination of bile salt tolerance

The effect of bile on the growth of strains was assessed by procedure of Walker and Gilliland with few modifications (Walker & Gilliland, 1993). In this assay 100 µL of bacterial strains at their exponential phase were inoculated in 10 ml of sterilized Tryptic soy broth (TSB, Oxoid ™ UK), similarly 100 µL of yeast strains at their log phase were inoculated in 10ml of sterilized Oxy-tetracycline glucose broth (OGB, Thermo Scientific ™ UK) present in the test tubes. Stock solution of salts of taurodeoxycholic acid and glycodeoxycholic acid (1g/10ml, Oxoid ™ UK) and lysozyme (0.01g/10ml, Thermo Fisher Scientific, USA) were prepared. In total 150µl of bile salt and 1ml of the lysozyme stock solution of was added in all the test tubes in order to have their final concentration as (1.5g/l) and (100µg/ml) respectively. The mixture pH was adjusted to 3.0 with HCl (1N). The bacterial samples were incubated at 37°C, while yeast samples were incubated at 30°C in shaker incubator at 150 rpm. Control were also prepared without adding microbial strains. The microbial growth was measured in terms of optimcal density by using spectrophotometer (Shimadzu, Japan) at 600nm after 2, 4, 6 and 24 hours interval. Bile tolerance of the isolates were calculated by using the following formula

% Survival = [OD of bile media / OD of control media] x 100

All experiments were done in triplicate.

3.2.3 Determination of cell surface hydrophobicity

The method used for this assay was the amended version initially devised by (Rosenberg *et al.*, 1980). The evaluation of the adherence capability of the microbes with the intestinal cell layer can be assessed by surface hydrophobicity test. Bacterial and yeast cultures were grown in the TSB and OGB media for 24 and 48hours respectively. Two mililiter of these cultures were and transferred micro-tube and centrifuge (Thermo Scientific™, USA) at 6000rpm for 5min to separate microbial cell pellet. The pellets was washed with normal saline (0.9% NaCl) followed by twice wahsings with phosphate buffer. The pellet was suspended in 3mL of autoclaved distilled water in separate test tubes after washing. Optical densities of these samples were measured at 600nm. Then 0.6mL of xylene was added into these tubes and vortex (Yelloline TTS 2, Germany) gently at 20 rpm to avoid foaming. After incubation at 37°C for 20-30 minutes, the aqueous layer optical density (600nm) was measured. The hydrophobicity was calculated by using the following formula:

Hydrophobicity (%) = [(A_0 – A_1) / A_0] x 100

Where,

A0= Optical density before mixing the xylene

A1= Optical density of aqueous layer

3.3 Determination of anti-Pathogenic activity of microbial isolates

After initially screening the selective isolates which were identified and which performed well in invitro screening tests were checked for antipathogenic acitivity in anticipation to their application in feeding trial. They were checked for their antimicrobial activity against the following pathogens *Staphylococcus aureus* (ATCC 2593), *Listeria monocytogenes* (ATCC 49594), *Salmonella enterica* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (25922). The supernatant was diluted according to 0.5 McFarland standard (Khunajakr, 2008) followed by lawn making with the help of sterile swabs on TSA plates and well was cut (6mm diameter) in agar plates. The 50 µl of supernatant after culturing tested bacteria and yeasts strains for 24 and 48 hours at 37°C and 25°C respectively in TSB

media. Zone of inhibition was checked after incubation of 24hours at 37°C (Magaldi *et al.*, 2004).

3.4 Antibiotic susceptibility of bacterial isolates

The antibiotic sensitivity of bacterial strains was tested after grown in the Tryptic soy broth (TSB) media for 24hrs at 37°C. Tryptic soy agar (TSA) plates were prepared and after complete incubation of bacterial strains a sterile cotton swab was dipped in to the bacterial culture and was rotated against the side walls of the test tube above the bacterial culture for the removal of the excess fluid. Dried Tryptic soy agar (TSA) plates were then swabbed three times over the whole agar plate for the even distribution of inoculum. The plates were then allowed to dry at room temperature for at least 3 to 5 minutes. The antibiotics used were vancomycin (VA30), Cefpirome (CPO30), Ampicillin (AMP25), Gentamicin (CN10), Ciprofloxacin (CIP5), Chloramphenicol (C30), Ceftazidime (Caz30), Ceftriaxone (CRO30) Piperacillin (pr1100) and Moxifloxacin (MXF5). The antibiotic discs were applied on the dried agar plates at appropriate distance with the help of forceps. Plates were inverted and placed in an incubator at 37°C for 24hrs. After complete incubation zones formed around the discs were measured (Bauer *et al.*, 1966).

3.5 Phase-II

Probiotic feeding trial on *L. rohita* under mimic aquaculture Conditions

3.5.1 Collection and acclimatization of fishes

Four hundred and sixty fingerlings of *L. rohita* having average body weight (5.90 ± 0.02 g) were purchased from commercial fish hatchery (Faisalabad Fish Hatchery Faisalabad-Pakistan) were transferred live in polyethylene bags that were fully aerated to the Fisheries and Aquaculture laboratory where water in the transportation bags was slowly replaced with non-chlorinated tap water for mitigation. A flow through system made of circular fiber tanks having efficient air stones for maximum supply of oxygen was used to retain the fish for the acclimatization period of two weeks. During this period *L. rohita* were reared by feeding 35% protein basal diet two times a day at the rate of 3% of their body weight.The fecal material and unexploited feed was siphoned off on regular basis. Monitoring of water quality parameters were done on regular basis, the parameters included the pH, dissolved oxygen and water temperature to make

assure that all these parameters were in accordance with optimum range. The natural day and night photoperiod was also monitored. The water temperature was kept at 25±1ºC for the *L. rohita* fingerlings throughout the experiment after the acclimatized *L. rohita* with identical size were shifted to 27 experimental glass aquaria.

3.5.2 Diet Preparation

The dehydrated feed ingredients as stated in Table 3.1, some of them were procured from the local market and the rest were obtained from National Agriculture Research Council (NARC) for preparing 35% protein basal diet for *L. rohita*. The ingredients were finely grinded and then mixed in fixed proportion.Vegetable oil was mixed and a dough was made with the addition of autoclaved distilled water and passed through the feed extruder.The resulting pellets were dried out at room temperature and were then stored in the refrigerator at 4ºC. The fresh feed was prepared on weekly basis for 90 days to maintain the original CFU of the probiotic microorganisms.

Table 3. 1*: Recipe of 35 percent protein basal diet for *L. rohita

Ingredients	Amount (g kg^{-1})
Soybean meal (46.2% CP)	212
Sunflower meal (40% CP)	212
White Fish meal (55%CP)	105
Gluten 30% (30% CP)	105
Canola meal (21.3% CP)	212
Rice polish (13.2% CP)	52
Dicalcium Phosphate	10
Carboxymethyl cellulose(CMC)	10
Vitamin premix[(a)]	20
Vegetable oil	10
Wheat bran	52

[a](Vitamin premix contains vitamins, amino acid and minerals premix kg^{-1})

CP, Crude protein, Manganese USP 30,000mg, Vitamin AB.P 40,000,000IU, Vitamin D3B.P 820,000IU, vitamin K3B.P 800mg, L. lysine B.P 10,500mg, Vitamin B2B.P 2500 mg, Vitamin EB.P 6200mg, Vitamin B12B.P 1000 mg, Vitamin B3B.P 5100 mg, Vitamin B.P 10,500mg, Choline chloride USP 125,500 mg, 15,100mg, Iodine B.P 300 mg, Copper B.P 1000mg, Zinc USP 17,555mg, Cobalt B.P 50mg, DL-Methionine B.P 50,500 mg.

3.5.3 Culturing and Preparation of Probiotic Feed

Probiotic cultures of bacteria were inoculated in the TSB broth and were incubated at 37°C for 24 hours while the yeast were cultured in OGB media and were incubated in shaking incubators (Thermoscientific, UK) for 48 hours at 28°C. Then the cultures were centrifuged at the speed of 10000 rpm (10 minutes at 4°C) to get metabolites as supernatant and cells, the extracted pellet was stored in separate tubes. The pellets were isolated and were sprayed through sterilized needles uniformly over the experimental diets with 35% protein. The feed was then partially dehydrated with the help of silica gel. In case of the control supplementation by probiotic cultures was not done. The probiotic cells were kept at a concentration of 10^{9}CFU gm^{-1}diet for both the yeast and bacterial cultures and was fed to *L. rohita* triplicate group of fish for 90 days. The cultures used in phase –II were selected on the basis of their probiotic screening, their ant pathogenic activity and on the basis of previously proven GRAS status.

3.5.4 Experimental design

Total 27 glass aquaria of dimensions (60 x 35 x 35 cm) were used for the 9 treatments applied on *L. rohita* having semi static conditions to perform the experimental trial. The groups made for the application probiotics in single form and consortia on *L. rohita* are shown in (Table 3.2). The stocking density of 1.5 g/L was retained where (n=10). For each group their particular probiotic supplemented diets were prepared by mixing it with the basal diets. Fishes were fed twice a day at the rate of 3% of their body weight. The undigested and fecal material was collected by draining off 20% of the water from each aquarium and was replaced with the same quantity of de-chlorinated water on daily basis.

Fish were fed with probiotic supplemented feed for 12 weeks. At the end of 12th week all the fishes were fasted for 24 hours before sampling. Fishes were anesthetized with a fatal dose of benzocaine (Sigma-Aldrich, Eastleigh, UK) followed by 100% ethanol swabbing prior to dissection from ventral surface.

3.5.5 Determination of Fish Growth performance

After forty-five and ninety days trial fishes weight was measured for the evaluation of specific growth rate (SGR), percentage weight gain (%WG), feed conversion efficiency FCE (%) and feed conversion ratio (FCR). Fishes were taken out from the aquaria and were anesthetized with MS-222 (60 mg L^{-1}) at the end of feeding trial. Then each fish was weighed and the blood was collected by the tail ablation. The fishes were dissected afterwards and GI tract was removed and were shifted to autoclaved Micro-tubes. All

the above mentioned parameters of growth performance were calculated via the protocol adopted by (Firouzbakhsh *et al.*, 2011; Wu *et al.*, 2012).

3.5.5.1 Calculation of weight gain

Initial body weight of *L. rohita* fingerlings was measured after the acclimatization period, which is used as initial body weight in equation. Final body weight was measured before dissection. Total percentage weight gain after 45 and 90 days trial was measured by using following formula.

$$\% \text{ Weight gain} = \frac{\text{Final body weight (Wf)} - \text{Initial body weight (Wi)}}{\text{Initial body weight (Wi)}} \times 100$$

3.5.5.2 Specific Growth Rate

Natural logarithm of final body weight and initial body weight was taken to calculate specific growth rate. The specific growth rate was evaluated by using the following equation.

$$\% \text{ SGR} = \frac{ln \text{ of final body weight } (ln \text{ Wf}) - ln \text{ of initial body weight } (ln \text{ Wi})}{\text{Duration of experiment (days)}} \times 100$$

3.5.5.3 Feed Conversion Ratio (FCR)

Feed conversion ratio was calculated by dividing total consumed feed by fish in during experimental trial and total weight gain of fish.

$$\text{FCR} = \frac{\text{Net consumed feed (g)}}{\text{Net wet weight gain (g)}}$$

3.5.5.4 Feed conversion efficiency (FCE)

Feed conversion efficiency was calculated by dividing feed conversion ratio by 1 and multiplying it with hundred.

$$\text{FCE (\%)} = \frac{1}{\text{FCR}} \times 100$$

3.5.7 Evaluation of the impact of probiotic feeding on fish hematological parameters

Blood samples that were collected from each treatment and control were processed for assessment of different hematological parameters such as RBCs, WBCs, Hemoglobin (HGB), Mean corpuscular hemoglobin(MCH), blood glucose and Hematocrit (HCT).

VACUETTE® EDTA K3 tubes were used to collect blood samples. These parameters are analyzed by using hematological analyzer (Sysmex KX-21N^{TM,} USA).

3.5.8 Assessment of blood glucose level

Blood glucose level was determined by using blood glucometer (ACCU-CHEK® Softclix, Canada). A drop of blood was added to the tip of glucometer strip and it was then inserted in glucometer. Glucometer displayed the glucose reading in mg/dL on the screen.

3.5.9 Determination of total plasma protein

The total protein content in blood plasma was determined by adopting protocol of (Lowry *et al.*, 1951). Different dilutions of Bovine serum albumin (Sigma Aldrich, Japan) were prepared followed by firstly ejecting 0.2 ml of protein solution from them and then shifting to the different test tubes.Finally 2ml of alkaline copper sulfate reagent was added to them. The solutions were mixed well and incubated at room temperature for 10 minutes. The tubes were incubated again for 30 minutes followed by the addition of 0.2ml of Folin Ciocalteau reagent (Sigma Aldrich, UK) to each tube. The absorbance was measured at 660 nm. A standard calibration curve was made by plotting absorbance against the protein concentration. The concentration of the unknown sample was determined by using the standard curve plot versus absorbance of unknown sample.

3.6 Determination of nutritional quality of fish

The crude fats and crude protein and ash content of the dried fish flesh were analyzed according to the standard protocols of (AOAC., 2000) from NARC, Islamabad. Crude fats and protein were measured using Soxthlet apparatus and micro kjeldahl method respectively (Sutharshiny & Sivashanthini, 2011).

The detailed procedure used for determination of body composition was as follows.

3.6.1 Dry Matter

Moisture content was measured by placing a washed china dish in hot air oven at 105°C for 15 minutes. Desiccation was done then it was weighed on digital balance. 5 g of sample was weighed again in that china dish and placed in oven for 24 hours at 105°C until constant weight was achieved. The china dish was cooled again in desiccator weight was measured again.

Formula used for determination of dry matter was:

% of dry matter = wt. of sample after drying / wt. of sample before drying x 100

3.6.2 Ash content

A crucible was washed and kept for an hour at 100 ºC in muffle furnace, then weighed after it cooled down. Then a sample of 2g was taken in the crucible and kept for 24 hours at 600 ºC in muffle furnace. Afterwards it was again kept in desiccator, and then allowed to cool and quickly weighed it before it absorbed moisture.
Following formula was used to determine the ash content:

Crude ash (%) = Weight of ash / Weight of sample x 100

3.6.3 Crude Protein

A small quantity of sample (almost 1 – 2g) was digested using conc. H_2SO_4 (30ml) and digestion mixture (5g) and heated on hot plate till the appearance of light green color at 250 ºC for 2 hrs. It was then allowed to cool. A solution was prepared in volumetric flask. Final volume was raised to 250ml by adding distilled water. 10ml of 40% solution was made and put in Kjeldahl apparatus and then was heated for 3 minute. 10 ml of boric acid (Sigma Aldrich_, Eastleigh, UK) (2%) was then added; released distillate (ammonia) was collected and using indicator (5 drops methyl red) was titrated against 0.1 N H_2SO_4. The process was continues till the appearance of yellow color.

%Nitrogen was calculated by using formula:

%Nitrogen = Normality of H_2SO_4 × Volume of H_2SO_4 used × 250 × 0.014 x 100 / Wt. of the sample × 10

Whereby;

250 = Dilution of the digested mixture

10 = Used volume of diluted mixture

0.014 = Standard volume of H_2SO_4 (0.1 N) to neutralize 1ml of

ammonia Percent Crude Protein = (%) Nitrogen x 6.25

3.6.4 Crude Fat contents

Soxthlet apparatus was used to determine crude fats employing hexane extraction method. Thimble was weighed and 2g of moisture free sample was taken in it. Thimble was positioned exactly under the condenser area of the apparatus. 150 ml hexane was taken in the receiving flask and it was connected with the apparatus. Heat and water were continuously supplied. The process of extraction continued for 2hours and 30 minute. Thimble was withdrawn, dried and reweighed.

Percentage of crude fats in the sample was calculated by using formula:

Crude Fates (%) = Weight of dried thimble after extraction – Weight of empty thimble / weight of the sample x 100

3.7 Determination of intestinal Enzyme contents

Six fish were collected from each aquarium after 45 and 90 days. The fishes that were anaesthetized with buffered MS-222 (60 mg L^{-1}) (Sigma Aldrich, UK) were dissected to remove digestive tract. 1g intestinal contents were homogenized with 10 ml of phosphate buffer (pH 7.5) using hand held glass homogenizer (Model AHS 200). The homogenate was then collected and centrifuged (Model Eppendorf centrifuge 5417R) at 15000 rpm and 4 °C for 15 min. The supernatant was removed and kept until analysis at 4 °C.

3.7.1 Determination of Protease Activity

For measuring the protease activity 0.65% casein (Sigma Aldrich, UK) solution (5 mL) was prepared. For preparation of the solution, 0.65g of casein was dissolved in water and incubated at 37ºC for 5 min. 1 ml enzyme solution was mixed with the above mentioned solution and then were further incubated at 37ºC for 10 minutes. The reaction was stopped by adding 5 mL of 110 mM trichloroacetic acid solution followed by re-incubation at 37ºC for 30 minutes (110 mM trichloroacetic acid solution was made by taking a volume of 55ml from 1 molar stock solution and adding 445 ml distilled water to make upto 500ml). The solution was then chilled to room temperature followed by filtration using Whatmann filterpaper (08µm). Almost 2 ml of filtrate was shifted in a 10 ml test tube followed by adding 500 mM Na_2CO_3 (5ml) and 0.5 mM Folin and Ciolcaltea's (1 ml).This mixture was then incubated for 30 minutes at 37 °C. It was allowed to cool and absorbance was determined at 660nm using UV-Visible spectrophotometer (Shimadzu, Japan).

3.7.2 Determination of Amylase Activity

Amylase activity was evaluated by using 3, 5-Dinitrosalicylic acid (DNS) (Sigma Aldrich, UK) method (Bernfeld, 1955) adopted by (Areekijseree *et al.*, 2004). The reducing sugar at 560nm was determined using maltose as the standard. 0.5 mL of enzyme solution was incubated at room temperature for 3 to 4 minutes. Then 1% starch solution (500 µL) was introduced and left for 3 minutes at ambient temperature (1% starch solution was prepared by dissolving 1g starch in 100 ml distilled water). To starch solution was added 1ml DNS reagent and incubated for 5 min on boiling water

bath followed by cooling at room temperature. Reagent grade water (10ml) was added and absorbance was determined at 540nm on spectrophotometer.

One amylase unit was defined as the amount of enzyme / mL filtrate that released one microgram reducing sugar / minute.

3.7.3 Determination of cellulase activity

The activity of cellulose, a digestive enzyme was determined using the methodology of (Denison & Koehn, 1977) with some modification. 2.932 g of sodium phosphate was mixed with 0.3708 g citric acid and added to 100ml H_2O to prepare citrate phosphate buffer of pH 5. 1%Carboxymethyl cellulase (CMC) solution was also added which was prepared by dissolving 1 g CMC in 100 mL of H_2O. Then reaction mixture was prepared containing 1 mL of CMC solution, 1 mL of appropriate enzyme solution along with 1mL of citrate buffer (0.1 M) was incubated for about half an hour at 50 ºC. Test tubes containing 3 ml DNS reagent were boiled for 15 min in water bath followed by incubation. 40% sodium potassium tartrate (1ml) was added to above mentioned test tubes and was kept for cooling at room temperature. Afterwards, the reducing sugar (glucose) which was produced from CMC substrate as a result of cellulolytic activity was determined on UV-Visible spectrophotometer at 540nm. One unit cellulase activity is defined as the amount of enzyme / mL culture filtrate that released 1 mg glucose / minutes.

3.8 Challenge test against *Staphylococcus aureus*

At the end of 90 days feeding experimental trial, two groups T0 (control) and T7 (*B.cereus* QAUBC02 and *G.candidum* QAUGC01) were selected for challenge test study. Each treatment was done in duplicate, having 6 fishes in each aquarium and observations were recorded for a week for any symptoms fish disease and mortality. Fishes were injected with 100µl of pathogen *S.aureus.*

3.9 Phase III

Study of impact of probiotic supplementation on fish gut Microbiology and Proteomics

3.9.1 Sample preparation

Fish gut samples from each treatment were taken after 90th day of dietary trial.

Intestinal collected samples that were stored at −20°C were further processed for the evaluation of microbial communities in the fish gut. Another one gram of intestinal sample was weighed by using weighing balance and were homogenized by adding 9ml phosphate buffer saline in sterile falcon tubes for culturable microbiological analysis. Centrifugation of mixture was done at 6000 rpm and for 5 minutes at 4°C, supernatant was collected in another sterile falcon for advanced analysis.

3.9.2 Determination of culturable microbial communites from fish intestine

For culture dependent analysis of microbial communities of fish intestinal sample, five different growth media were used. Tryptic soya agar (TSA) was used as a general purpose or non-selective media, estimating an overall bacterial count. Among selective media, M17 for enumerating *Enterococcus* sp, de Man Rogosa Sharpe media (MRS) (pH 5.4) was used for enumerating *Lactobacillus* sp., *Streptococcus* sp. and *Lactococcus* sp. MacConkey agar for enumerating the members of Enterobacteriaceae and Oxytetracycline glucose agar (OGA) media for the enumeration of yeast species(David *et al.*, 2014) . All the media were purchased from Oxoid. *Nilstat* (Nystatin) antifungal agent was added to avoid fungal contamination in bacterial media, while in OGA Oxy-Tetracycline Dehydrate was added as antibacterial agent to avoid bacterial contamination.

All media were prepared according to the manufactures' recommendation sterilized by autoclaving at 121°C at 15lb pressure for 15 min. Plates were prepared by pouring the media aseptically in bio safety hood.

Previously prepared samples were serially diluted by doing 10-fold dilutions up to 10^{-7}. 100µl sample from dilutions 10^{-4}, 10^{-5} and 10^{-6} were inoculated by micropipette on the plates. Spreading of inoculum was done by using sterile glass spreader, which was sterilized after each sample by dipping in ethanol followed by heating on flame. The plates for bacterial culture were incubated at 37°C while plates for yeast culture were incubated at 30°C for 24-48 hours followed by spreading.

3.9.3 Macroscopic examination

After 24-48 hours incubation, macroscopic or morphological characteristics of colonies were observed. Colony morphology, size, color, margins were recorded.

3.9.4 Colony forming unit (CFU/g) count

Number of colonies on each plate was counted by using colony counter (Boeco, Germany) as per standard protocol. Then the number of colonies was converted into

CFU or log value/g by using the CFU calculation formula, to assess the cultivable microbiome of fish gut.

CFU/g = (No. of colonies x dilution factor) ÷ volume plated in ml

3.9.5 Microscopic examination

The cultivable microbiomes of gut were also examined microscopically followed by macroscopic examination. Bacterial colonies from all four types of media were examined by using Gram staining standard protocol, while yeast colonies were examined by using lacto phenol cotton blue staining.

3.9.5.1 Gram's staining

Small drop of saline was placed on a glass slide. With the help of loop, bacterial colony was picked and mixed with saline to form a thin smear. After air drying, the smear was heat fixed. The smear was stained with crystal violet (for 60 sec) and rinsed with distilled water. Then two drops of Gram's Iodine was added (for 45 sec), and again rinsed with distilled water. After that, 2 – 3 drops of 95% ethanol were used as decolorizer. Finally, safranin was added (for 45 sec) and rinsed. The slide was air dried and observed under the microscope using oil-immersion at 100X.

3.9.5.2 Lactophenol cotton blue staining

Smear of yeast culture was prepared on a clean glass slide and air dried. Few drops of lacto phenol cotton blue stain was added and covered with a clean cover slip. Slides were observed under oil immersion lens at 100X.3.10 Determination of gut microbiology by culture independent method (16S rRNA based Metagenomics).

3.10.1 DNA extraction

Favor Prep Stool DNA Isolation Mini Kit (FAVORGEN) was used to extract DNA according to their mentioned protocol. The 100mg of frozen intestinal sample was taken in 2ml Micro-tube and 200mg glass beads were transferred in the tube, 300µl SDE1 buffer and 20µl proteinase-K were also added in the sample for lysis of cells. The mixture was then vortexed at maximum speed for 5 minutes and incubated at 70°C for 10 minutes followed by 5 minutes more incubation at 95°C (for Gram positive bacteria). During incubation samples were vortexed twice to homogenize the intestinal content completely. Drops from the lid were removed by spinning the samples briefly. Samples temperature was lower down, 100µl SDE2 buffer was added to the samples

and vortexed to mix it well. After 5 minutes incubation on ice pack samples were centrifuged by using bench top centrifuge at 14,000 rpm and 4°C for 5 minutes. Supernatant was transferred to another set of micro-tubes carefully and 200µl SDE3 buffer was added. The mixture was vortexed to mix it and incubated at room temperature for just 2 minutes. Samples were centrifuged at 14, 000 rpm and 4°C for 2 minutes, supernatant was transferred to the next clean micro-tubes and pellet was discarded. 1µl RNase of concentration 100mg/ml was added in the supernatant and mix well to avoid RNA contamination. Brief spinning of the tubes was done to remove drops from lid. 250µl SDE4 buffer and 250µl chilled ethanol was added in the above mixture mix carefully by pulse vortexing. Columns were placed in the collection tubes and transfer the mixture to the column. The samples were centrifuged at14,000 rpm for 1 minute, columns were transferred to next clean collection tubes and flow-through was discarded.750µl of wash buffer was added to columns and centrifuge them as above-mentioned conditions for 1 minute. The washing step was repeated followed by 3 minutes additional centrifugation to dry the columns and avoid residuals contamination which may inhibit enzymatic reactions. Columns were transferred into elution tubes; 50-200µl elution buffer was added exactly in the center of the columns to elute down the DNA. 2 minutes incubation was given to the columns that elution buffer can absorbed in membrane completely and then samples were centrifuged to elute down DNA. For further analysis the extracted DNA samples were stored at −20°C.

3.10.1.1 Gel electrophoresis

Qualitative confirmation of extracted DNA was done by using gel electrophoresis. For this purpose 1% gel was prepared by adding 1g agarose gel (Sigma Aldrich™) in 10ml TBE buffer and 90 ml distilled water. The gel was heated in microwave for 30 seconds twice to mix it completely, temperature was lower down until it become bearable. 4µl ethidium bromide was added as an indicator to visualize DNA bands. Gel was poured in the casting tray having comb and allowed the gel to solidify gel 10 to 15 minutes at room temperature.

Gel casting tray was transferred to the gel tank having 1X TBE buffer and comb was removed carefully.2µl bromophenol blue dye with 3µl sample was added, mixed thoroughly by using micropipette and loaded carefully in wells. The sample was run on gel at 120 volts for 30 minutes and 400mA. Gel was observed by using UV illuminator after 30 minutes and recorded the results. The samples having DNA stained with

bromophenol blue illuminate in UV. Extracted DNA purity and quantity was determined by Nano Drop1000 spectrophotometer (Thermo scientific).

3.10.2 Metagenomics analysis

To produce the fragment of 16S rRNA gene V4 variable region of each sample PCR was performed.The primers 515F (GTGCCAGCMGCCGCGGTAA) / 806R (GGACTACHVGGGTWTCTAAT) were used (Caporaso *et al.*, 2011) and PCR was run in three replicates in 20µL reaction volume. The 20µL reaction mixture was prepared by using the HotStar TaqPlus Master Mix Kit (Qiagen, USA) and sample. The conditions for PCR was as follows initial denaturing, 94°C for 3 min, which then followed by 30 cycles denaturation at 94°C for 30 seconds, annealing at 53°C for 40 seconds and extension at 72°Cfor 1 minute. After 30 cycles final elongation was performed at 72°C for 5 minutes. The amplified PCR products were tested in 2% agarose gel to determine the accomplishment of amplification and the relative intensity of bands. After determination, all samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified by using calibrated Ampure XP beads, Libraries were prepared by using these polled purified PCR products by using Nextera DNA sample prep kits and following the Illumina Tru seq DNA library preparation protocol. Miseq sequencer was used for sequencing analysis as per manufacturer's instructions at MR DNA (Shallowater, TX, USA) following the manufacturer's guidelines. Statistical and Bioinformatics Analysis of sequenced data were performed by using MR DNA analysis pipeline (MR DNA, Shallowater, TX, and USA). Sequences were joined, depleted of barcodes to reduce the sequencing error. Sequences with < 150bp, with ambiguous base calls, with homopolymers were removed for accuracy.Then the final sequences were denoised, Operational taxonomic units (OTUs) were produced and chimeras detached. Operational taxonomic units (OTUs) were further defined by clustering at 3% divergence (similarity 97%). Final operational taxonomic units (OTUs) were classified taxonomically using BLAST against curated database derived from GreenGenes, RDPII and RDPI (DeSantis *et al.*, 2006).

Metagenomic sequences will be annotate using evidence based annotation approach. Sequences will be Blast against protein databases at an E-value cutoff and predicted genes will be organized and categorized into functional groups from lower orders (individual genes) to higher orders (cellular processes). Relative abundance for each

gene will be calculated by dividing the similarity hits for an individual gene by total hits against any of the database. To comprehend the gradient specific functional traits, Megablast was used to understand the endemic metagenomics reads (Reads of one metagenome against combination of remaining).

3.11 Proteomic analysis of Fish intestinal contents

3.11.1 Sample preparation

Fish intestinal contents samples from the treatments produced maximum beneficial effects on physiological parameters were taken along with inner intestinal mucosa and lyophilized for further proteomic analysis.

3.11.2 Protein digestion

The concern band was removed from the gel and was shifted to micro tube of 0.5 ml. The piece of gel was poured with 200 µL of solution (Acetonitrile mixture, ammonium bicarbonate and, 2.5 mL bicarbonate of ammonium, 1M of 47.5 mL water and generate the volume to 100 ml with acetonitrile) for approximately 30 minutes. According to color consecutive coatings with bicarbonate of ammonium 25 mM and solution A was repeated. Later the matrix retrained wash using about 200 ul water from ultrapure HPLC and with 100% acetonitrile. Every coating took 15 minutes for completion along with agitation at room temperature. Supernatant was pipetted out and gel was dried on vacuum pump specifically for 5 minutes at room temperature. Diluted solution of trypsin (0.006 µg/µL in 25 mM ammonium bicarbonate) up to 25 µL was supplemented in gel portion and hydration was done for the next 15 minutes inside ice. 30µL of 25 mM bicarbonate of ammonium was used for whole hydration, and was agitated at 37°C for a night. Supernatant was removed afterwards, all peptide residues were taken out using 50% of acetonitrile along with 100% acetonitrile and 5% formic acid. In the gel piece, 30 µL of single extracting solution was added and then incubated and agitated for 15 min monitored by desiccation in vacuum pump or on the burning plate at 55°C till no liquid remained. The taster was kept at -20 °C till examined.

3.11.3 Identification of proteins

Peptide residues were dissolved in 10 µL of 1 % trifluoroacetic acid (TFA), then 1 µL of solution remained was mixed in 1 µL of α- cyano -4-hydroxycinnamic acid (CHCA). The peptides were identified through MALDI TOF/TOF (Matrix assisted laser desorption ionization –time of flight) (mass spectrometry). Records base searches were

approved with MS/MS ion search (MASCOT, http://www.matrixscience.com) software.

An irregular gel of 1D polyacrylamide was set holding a gel of 4% concentration which allowed the facilitated scattering of the proteins at the border of the second gel that is 12% separation gel warranting the departure of the proteins as a function of the molecular weight. Both gels of different concentrations of polyacrylamide were superimposed on each other. This solution was obtained by mixing all the reactants in a vacuum flask, except TEMED and APS. The protein tester left in the well was mixed with Laemmli buffer and heated at 95°C for 2 min. The Laemmli buffer was prepared of SDS and Bromophenol blue to which β-mercaptoethanol must be added. The dye was used to observe the movement of proteins throughout electrophoresis was BBP. The height of the gel was 6.8 cm, thickness was 0.75 mm, and a width was 8.6 cm. The electrode buffer (pH 8.3) consists of 384 mM glycine, 50 mM Tris, and 0.1% SDS. A volume of 20 µL of Laemmli buffer (50 µl of 2-mercaptoethanol added to 950 µL of Laemmli Bio-rad solution) was added to 20 µL of tester (protein solution), at that moment 20 µl of the preparation are deposited in the gel as a form of well. The molecular weight markers used is Protein Ladder PiNK Prestained had 10 proteins which resolve into tight net bands of the order of 15-175 kDa. Later the migration, the gel was mixed with proteins with dehydration solution(10% acetic acid, 50% ethanol) for almost 10 min at 55°C. Afterwards this gels are kept in a staining solution (7.5% acetic acid, 5% ethanol) and a Coomassie blue dye was applied after heating for around 10 min at 55°C.

3.11.3.1 Preparation of the sample for identification with MALDI TOF-TOF

3.11.3.2 Digestion of Trypsin Proteins

The washing a tryptic digestion of the samples obtained from gels stained by coomassie blue was done to create a peptide mass imprint in demand to detect the proteins by TOF/ MALDI-TOF mass spectrometry. The protein spots (placed in 0.5 ml micro-tubes) was washed in order to eliminate the part of the remaining and coloring salts. Later, the tasters were break down by a very active enzyme: trypsin (cleavage of the C-terminal side in the amino acids basic lysine and arginine). Then on a metal plate this digestive supernatant was deposited with a matrix (CHCA) and put in the MALDI source to be identified and analyze the protein in a database. Preparation of solutions:

- Stock solution of 1M ammonium bicarbonate
- 5 % Formic acid
- 50% Acetonitrile
- 25 mM ammonium digestion buffer solution
- Solution A containing 25 mM bicarbonate and 50%
- acetonitrile 0.4 µg / µl trypsin solution

There were many phases of preparing a piece of gel through an extraction of the alternating peptides of acetonitrile and formic acid will take place. Initially, the gel strips were set to split into sections and kept in micro-tubes for washings.

3.11.3.3 Washings

The initial wash was performed with about 200 µl of solution A for 30 min (cover the solution spot). Depending on coloration, a continuous repetition of wash with bicarbonate of ammonium and solution A was performed. As soon as the protein dot had been decolorized, washing with 200 µl of Ultrapure HPLC water at room temperature was done for about 15 min and through stirring. On the same pattern, further washing with 100% acetonitrile is performed. After washing, the supernatant was removed with the help of pipette and then, the gel was dried for 5 min at speed vac at room temperature. Afterwards, for few seconds, water was vortexed and around 200 µl of Ultrapure was added. After the removal of supernatant about 200 µl of 100% acetonitrile was added, which was kept to react for total 15 min with continuous stirring at room temperature. Again the supernatant was detached and the gel bits were dried out at room temperature at Speed Vac for 5 min.

3.11.3.4 Digestion of soluble peptides

A 0.4 µg / µL trypsin solution was present which is diluted to 0.006 µg / µl with 25 mM bicarbonate of ammonium. 10 µL of trypsin was added to 665 µl of 25 mM bicarbonate of ammonium. After the solution was ready, 25 µl of this solution was added to every tube containing the cut, decolorized and dried out protein dots in command to rehydrate gel for about 15 min at 4°C. The pieces of gel were fully hydrated by adding 30 µl of 25 mM bicarbonate of ammonium to achieve excessive buffering and then to be incubated for almost 30 min at 37°C with regular stirring followed by overnight incubation in oven at 37°C.

3.11.3.5 Method of extraction of the peptides in alternation

After performing hydration of gel, the digestive supernatant was moved to the other tube where, for at least 15 minutes 30 µl of 50% acetonitrile has to be supplemented in the medium (gel). The recovered supernatant was added in to digestive supernatant. Around 30 µl of 5% of a formic acid was added to the left over piece of gel and permitted to extract for15 minutes. In the preceding supernatants again the recovered supernatant was added. 100% acetonitrile was once again added to tubes holding the gels and kept for 15 minutes.When the supernatant was recovered then it is added in to the preceding supernatants. The supernatants were dried at Speed Vac.The protein pellet was re-suspended in 10 µl of the buffer A (0.1% TFA) and a sonication for few seconds followed by a vortex was applied. A microliter of buffer A was then added to 10 µl of alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix buffer and then stirred. Afterwards, a microliter of mixture was set down on the Maldi plate made up of stainless steel Opti-TOF 384 targets, before introduction into the mass spectrometer, the protein droplet deposited on the plate was evaporated.

MS (mass spectrometry) experiments were carried out by using proteome analyzer AB Sciex 5800 equipped with an optical TOF/TOF and an axis on an OptiBeam TM laser radiation with a replication rate of 1000 Hz. Instantaneously the equipment was standardized prior the examination with a mixture of Angiotenin I, des-Arg-Bradykinin, ACTH (7-38), ACTH (18-39) (LaserBio Labs, Glu1-Fibrinopeptide B, Sophia Antipolis, France). All acquisitions were made under programmed mode. For ionization, laser intensity 3000 is mostly used. In the positive reflector mode the MS spectra were acquired by brief 1000 unique spectra in the 600-2000 DA mass range. The results attained by mass spectrometer are indispensable for the analysis of basic data through the Mascot software.

3.12 Statistical Analysis

The data was analyzed statistically by using XLSTAT 2014.5.03. Factors selected for statistical analysis were invitro characterization for probiotics screening, growth performance, hematological, biochemical parameters. Pearson Correlation was applied to determine the correlation between different parameters such as probiotic organisms, hematological parameters, gut microbiota, enzymes activity and growth performance. Analaysis of variance (ANOVA) followed by Tukey's and Duncan's analysis was also applied to check variance between different treatments. An ANOVA statistical analysis was executed for the comparison of the growth of strains.

04. RESULTS

04. RESULTS

4.1 Isolation and identification of microbial isolates from fish gut

4.1.1. Microscopic and biochemical characterization of isolates.

Isolation of the fish instestinal microbiota was done both from wild and farmed fishes. The isolated colonies obtained only on TSA media while no growth was observed on other three media MRS, M17 and OGA. The selected colonies were further purified on respective meida and for saved in 20% glycerol at -80°C. Microscopy of selected isolates showed that 11 out of 12 strains were Gram positive cocci and one was Gram positive rod. The results of microscopic and biochemical characterization are shown in Table 4.1.

***Table 4. 1*: Microscopic examination and biochemical properties of bacterial isolates from fish intestinal contents**

Sr. no.	Microbes (Lab codes)	Gram staining	Oxidase	Catalase
1	F01	G +ve cocci	-ve	-ve
2	F03	G +ve rods	-ve	-ve
3	F07	G +ve cocci	-ve	-ve
4	F08	G +ve cocci	-ve	-ve
5	F18	G +ve cocci	-ve	-ve
6	F19	G +ve cocci	-ve	-ve
7	F20	G +ve cocci	-ve	-ve
8	O1	G +ve cocci	-ve	-ve
9	O2	G +ve cocci	-ve	-ve
10	O14	G +ve cocci	-ve	-ve
11	O20	G +ve cocci	-ve	-ve
12	O29	G +ve cocci	-ve	-ve
12	F19	G +ve cocci	-ve	-ve

Enterococcus hirae* QAUF01(F01), *Bacillus cereus* QAUBC02 (F03), *Enterococcus faecium* QAUF18 (F18), *Enterococcus mundtii* QAUF20 (F20) are identified by 16S rDNA from gut of *L. rohita* while (O1,O2,O14,O20,O29,F07 and F08) are unidentified isolates from fish gut. **+ve (positive)*-ve (negative).

4.2 Molecular identification of isolates

Based on alignment of 16S rDNA sequence of the bacterial strains, phylogenetic tree shows their evolutionary origin. Searching the NCBI database against all available bacterial genome sequences, the bacterial strains from fish gut F03, F01, F18 and F20 displayed high sequenced belong to the *Bacillus cereus* QAUBC02, *Enterococcus hirae* QAUF01, *Enterococcus faecium* QAUF18 and *Enterococcus mundtii* QAUF 20 (Figure 4.1-4.4). The dendogram based on FTIR analysis give the clustering pattern of isolates F7, F8, F19, F20,O1,O2,O14 and O29 (Figure 4.5)

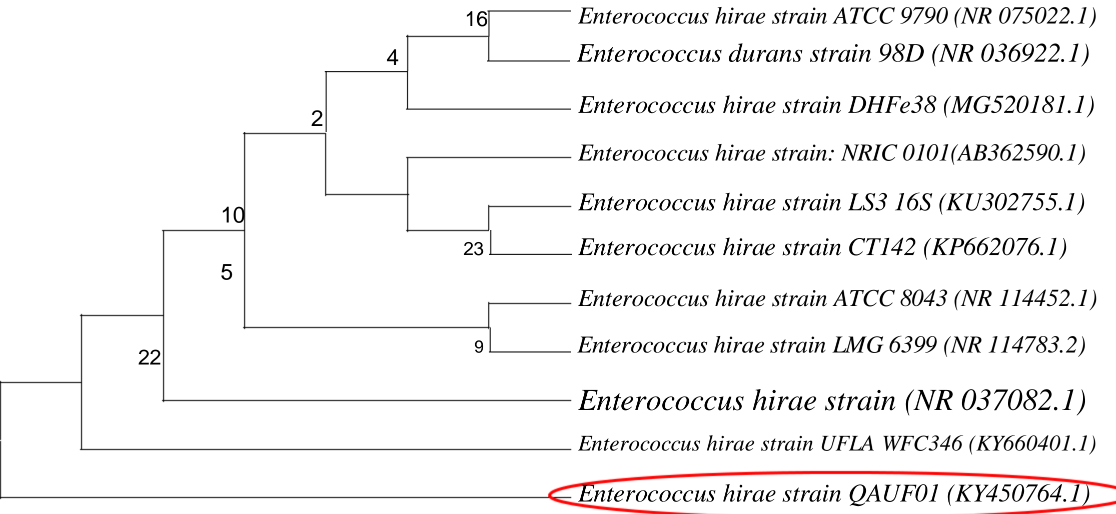


Figure 4. 1: **Phylogenetic tree based on 16S rDNA sequences showing the position of bacterial isolate F01 derived from fish gut having close resemblance with *Enterococcus hirae* QAUF01. The tree was constructed in MEGA6 by the neighbour-joining method derived from the 16S rDNA sequences.**

***F01represents *Enterococcus hirae* QAUF01**

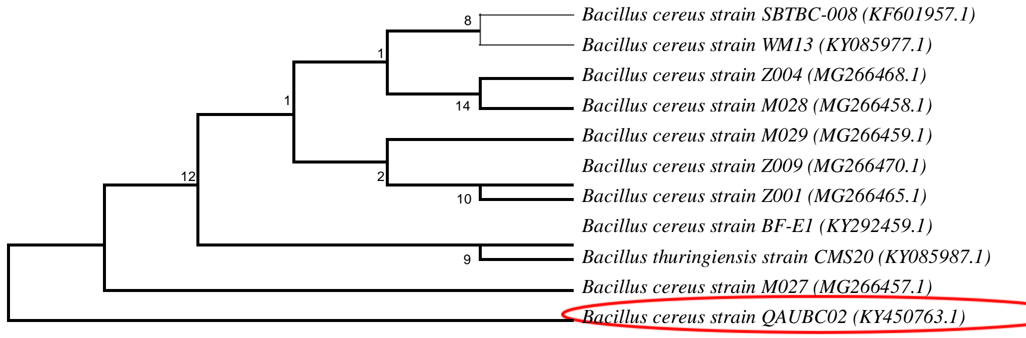


Figure 4. 2: **Phylogenetic tree based on 16S rDNA sequences showing the position of bacterial isolate F03 derived from fish gut having close resemblance with *Bacillus cereus* QAUBC02. The tree was constructed in MEGA6 by the neighbour-joining method derived from the 16S rDNA sequences.**

***F03 represents *Bacillus cereus* QAUBC02**

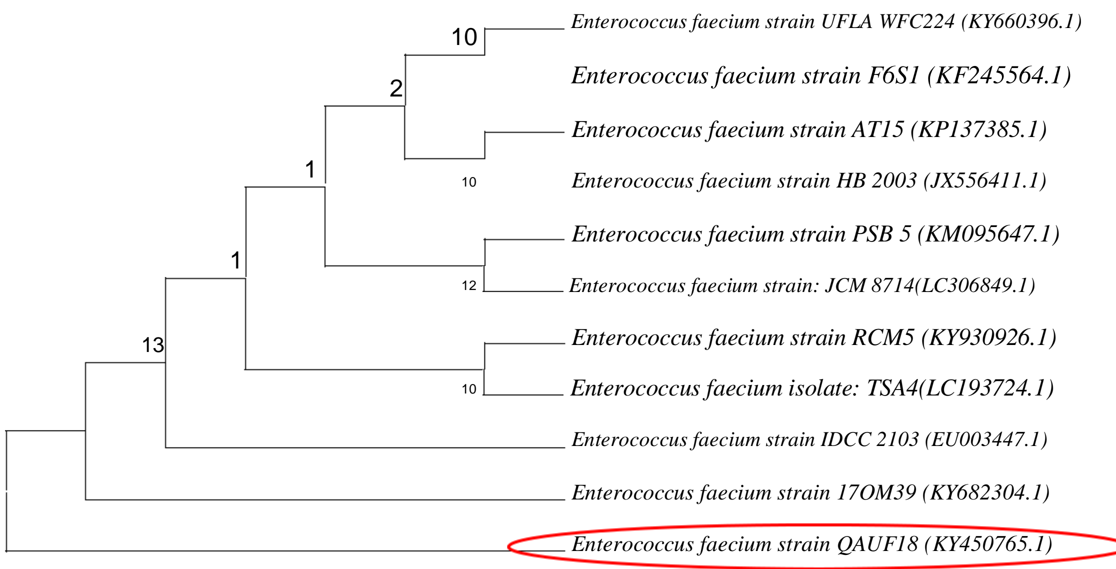


Figure 4. 3: **Phylogenetic tree based on 16S rDNA sequences showing the position of bacterial isolate F18 derived from fish gut having close resemblance with *Enterococcus faecium* QAUEF18. The tree was constructed by the neighbour-joining method in MEGA6 derived from the 16S rDNA sequences.**

***F18 represents *Enterococcus faecium* QAUF18**

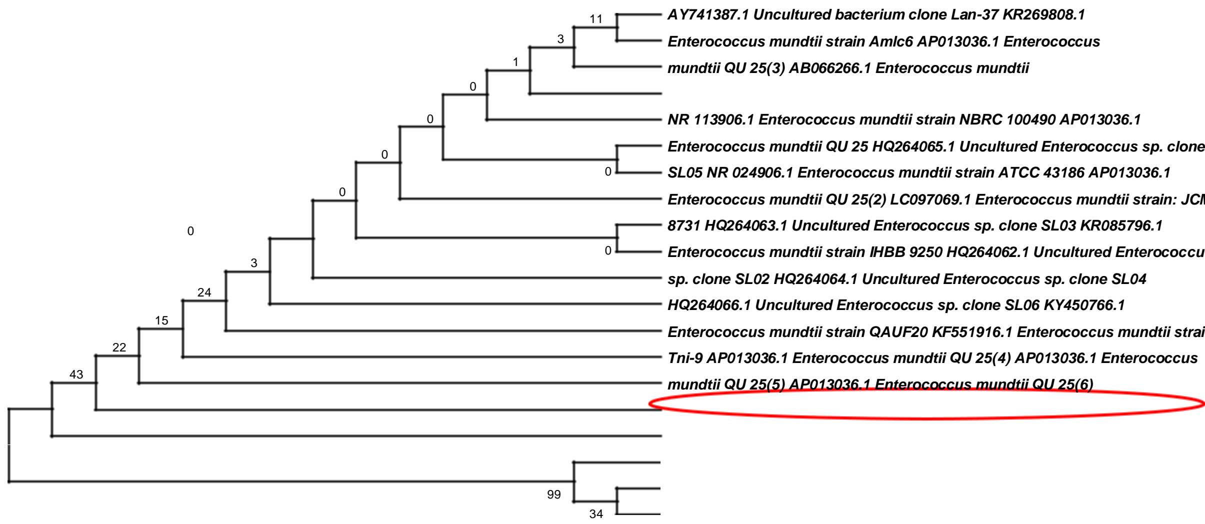


Figure 4. 4: Phylogenetic tree based on 16S rDNA sequences showing the position of bacterial isolate F20 derived from fish gut having close resemblance with **Enterococcus mundtii** ***QAUF20. The tree was constructed by the neighbour-joining in MEGA6 method derived from the 16S rDNA sequences.***

***F20 represetns *Enterococcus mundtii* QAUF20**

4.3 Determiniation of identification of fish gut isolates based on FTIR analysis.

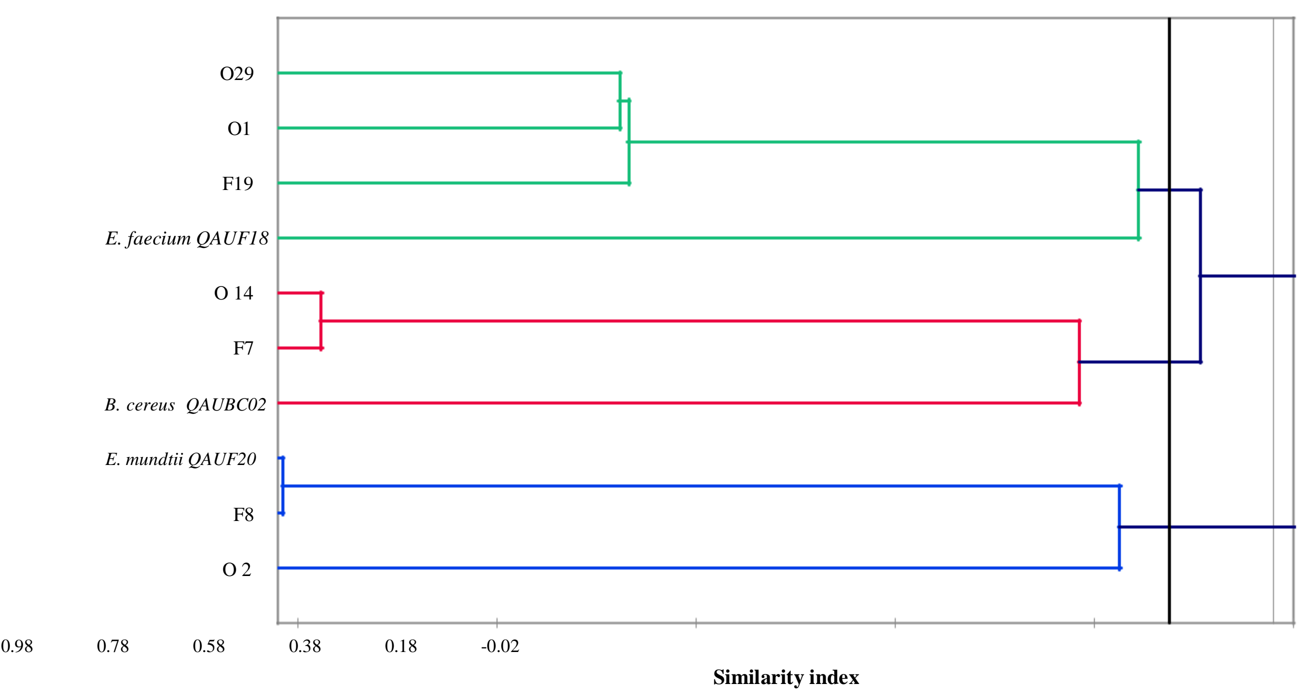


Figure 4. 5 **: Dendogram of fish gut isolates by FTIR analysis based on similarity index.**

In vitro characterization of isolates and co-cuture of isolates were performed .

4.3.1 Acid tolerance of the isolates

The acid tolerance of all strains were tested by maintaining the initial media pH at 2, 5, 7 and 9 showed the following results. The maximum growth at pH 2 was observed by *E. faecium* QAUEF01 (0.539±0.0052) followed by O14 (0.510±0.005), F7 (0.478±0.009), F19 (0.453±0.0036), O2 (0.44±0.003) all other isolates and combination of isolates didn't showed significant growth at pH 2. The strains F7 and F19 vary non-significant with respect to each other. O1, *G. candidum* QAUGC01, *G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02 vary non-significantly with respect to each other and minimum growth was observed for them (0.073±0.0009), (0.066±0.0002) and (0.101±0.017) respectively. *G. candidum* QAUGC01 combined with *E. faecium* QAUEF01 and F8 were also non-significant variant with respect to one another.

Maximum growth at pH 5 was observed by *E. mundtii* QAUF20(0.956±0.007) followed by *E. faecium* QAUEF01 (0.932 ±0.0038), F07 (0.884±0.007), O14 (0.8660 ±0.0039), *E.*

faecium QAUF18 (0.846±0.014). At pH 7 maximum growth was observed by F8 (1.84±0.002), followed by O1 (1.776±0.007), *E. hirae* QAUEH01 (1.732±0.007), F19 (1.573±0.006) QAUGC01 showed minimum growth with value (0.0976±0.00038).

At pH 9 maximum growth was observed by O14 (1.317±0.0067) followed by F19 (1.160±0.0169), *E. hirae* QAUEH01 (0.8634±0.0027), *E. mundtii* QAUF20 (0.828±0.0045), minimum value in terms of growth was observed by O29 (0.1190±0.0049) (Figure 4.6, Table 4.2)

Survival of the bacterial isolates O1, O2, O29, *E. mundtii* QAUF20, *E. faecium* QAUF18, F7, F8, F19, O14, *E. faecium* QAUEF01, *E. hirae* QAUEH01, *B. cereus* QAUBC02, *G. candidum* QAUGC01 (yeast isolate) and combination of *B. cereus* QAUBC02 and *G. candidum* QAUGC01, combination of *E. faecium* QAUEF01 and *G. candidum* QAUGC01, combination of *E. hirae* QAUEH01 and *G. candidum* QAUGC01 were observed after 2 hours, 6 hours and 24 hours by comparing the growth rates of bacterial, yeast strains, combination of yeast and bacterial isolates in a media having pH 3, bile concentration 1.5g/L and lysozyme concentration 100µg/ml with the control media possessing neutral pH that is 7 with no lysozyme enzyme and bile salts.

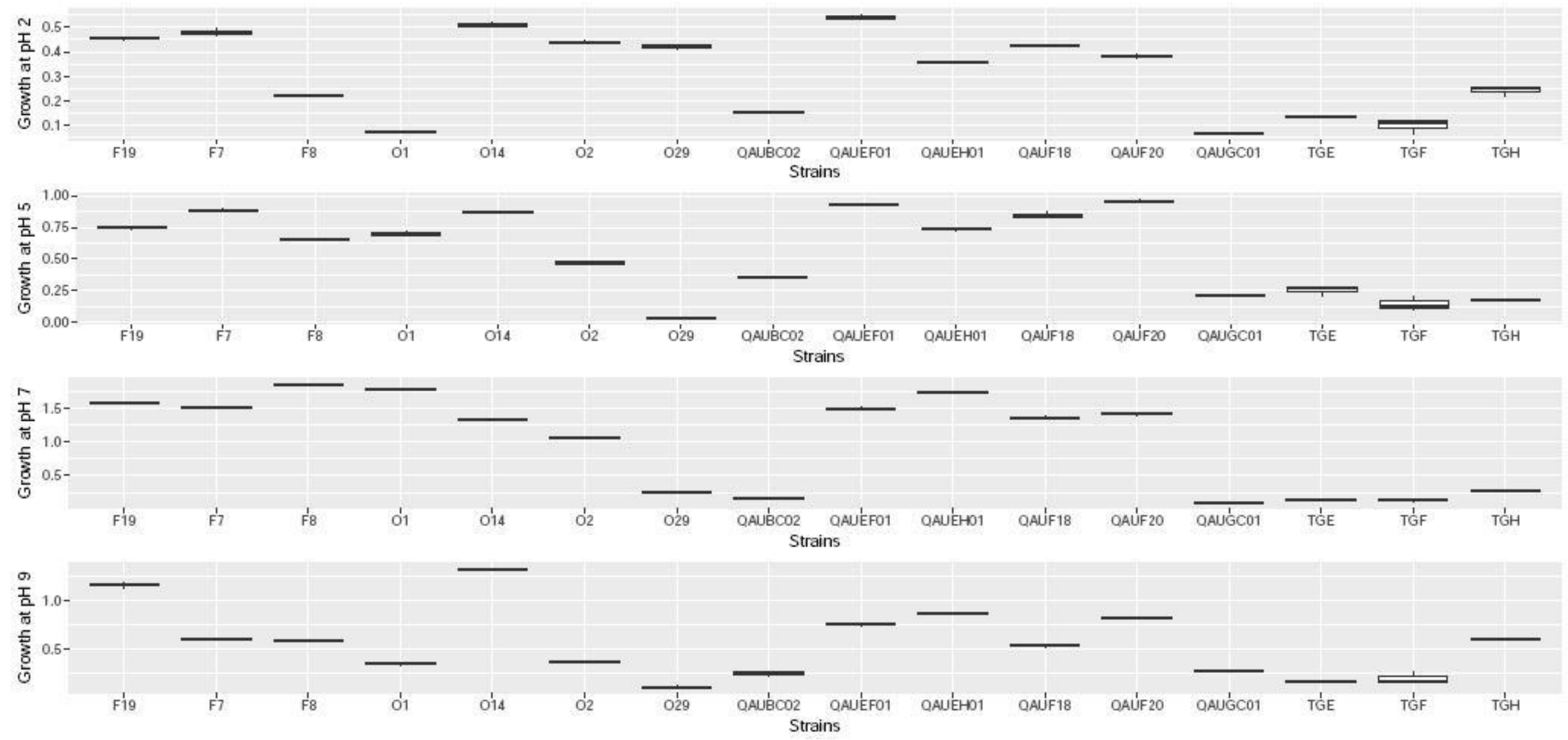


Figure 4. 6**: Acid tolerance at different pH by taking relative growth (OD at 600nm) .** ***This is the box plot graphical respresentation of data generated in statistical R software..,******QAUF20 (*****E. mundtii*** **QAUF20),*QAUF18 (*****E. faecium*** **QAUF18), *QAUBC02 (*****B. cereus*** **QAUBC02), O1, O2, O29, F7, F8, F19, O14 are unidentified fish gut isolates, TGF [*****G. candidum*** **QAUGC01 and** ***B. cereus*** **QAUBC02], TGE(*****G. candidum*** **QAUGC01** ***and E. faecium*** **QAUEF01, TGH (*****G. candidum*** **QAUGC01 and** ***E. hirae*** **QAUEH01), QAUGC01 (*****G. candidum*** **QAUGC01), QAUEF01(*****E. faecium*** **QAUEF01) , QAUEH01(*****E. hirae*** **QAUEH01). This graph is the mean of three independent experiments .**

After 2 hours maximum survival was observed by *B. cereus* QAUBC02 (91.47±1.61) followed by *G .candidum* QAUGC01 and *B. cereus* QAUBC02 combination (88.54±4.73), F19 (87.64±1.76), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 combination (82.31±5.35), O1 (78.42±3.14), F7 (58.21±0.32), *E. faecium* QAUEF01(54.36±1.94), *E. hirae* QAUEH01 (51.16±4.62), *E. faecium* QAUF18 (51.06±3.15), *E. mundtii* QAUF20 (44.36±2.01). The remaining single isolates and the combination showed less than 50% survival rate including *E. hirae* QAUEH01 and *G. candidum* QAUGC01 combination (43.11±3.66), *G. candidum* QAUGC01 (38.66±0.57), O14 (37.42±2.84), O2 (33.58±1.51), F8 (31.82±0.62) and O29 (27.48±1.94).

The maximum survival after 6 hours was observed by *G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture (89.71±1.49), *B. cereus* QAUBC02 (85.72±1.27), O1 (87.05±5.26), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 combination (75.30±2.89), F8 (72.83±1.59), F7 (72.27±3.14), O29 (71.42±4.09), *G. candidum* QAUGC01 in combination with *E. hirae* QAUEH01(70.98±1.21),O2 (68.36±3.35), *E. faecium* QAUEF01 (46.39±4.94), *E. hirae* QAUEH01 (46.34±1.39), F19 (43.99±3.05), *E. faecium* QAUF18 (41.47±2.47), *G. candidum* QUAGC01 (36.30±1.62), O14 (36.45±0.48), *E. mundtii* QAUF20 (29.63±3.21) respectively.

The maximum survival after 24 hours was observed by F7 (89.97±3.72) followed by F19 (89.57±0.42) , *B. cereus* QAUBC02 (85.01±1.85), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 combination (84.81±1.42), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 combination (79.10±2.13), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 combination (76.3801±0.65), O1 (66.09±3.66), F8 (51.89±1.77), *E. hirae* QAUEH01 (43.25±0.92), O29 (42.96±0.53), O2 (37.05±2.53), *G. candidum* QAUGC01 (34.73±2.17), *E. faecium* QAUF18 (34.43±4.57), O14 (34.03±0.54), *E. faecium* QAUEF01 (32.83±0.28) and *E. mundtii* QAUF20 (27.80±1.39) respectively (Figure 4.7 and Table 4.2).

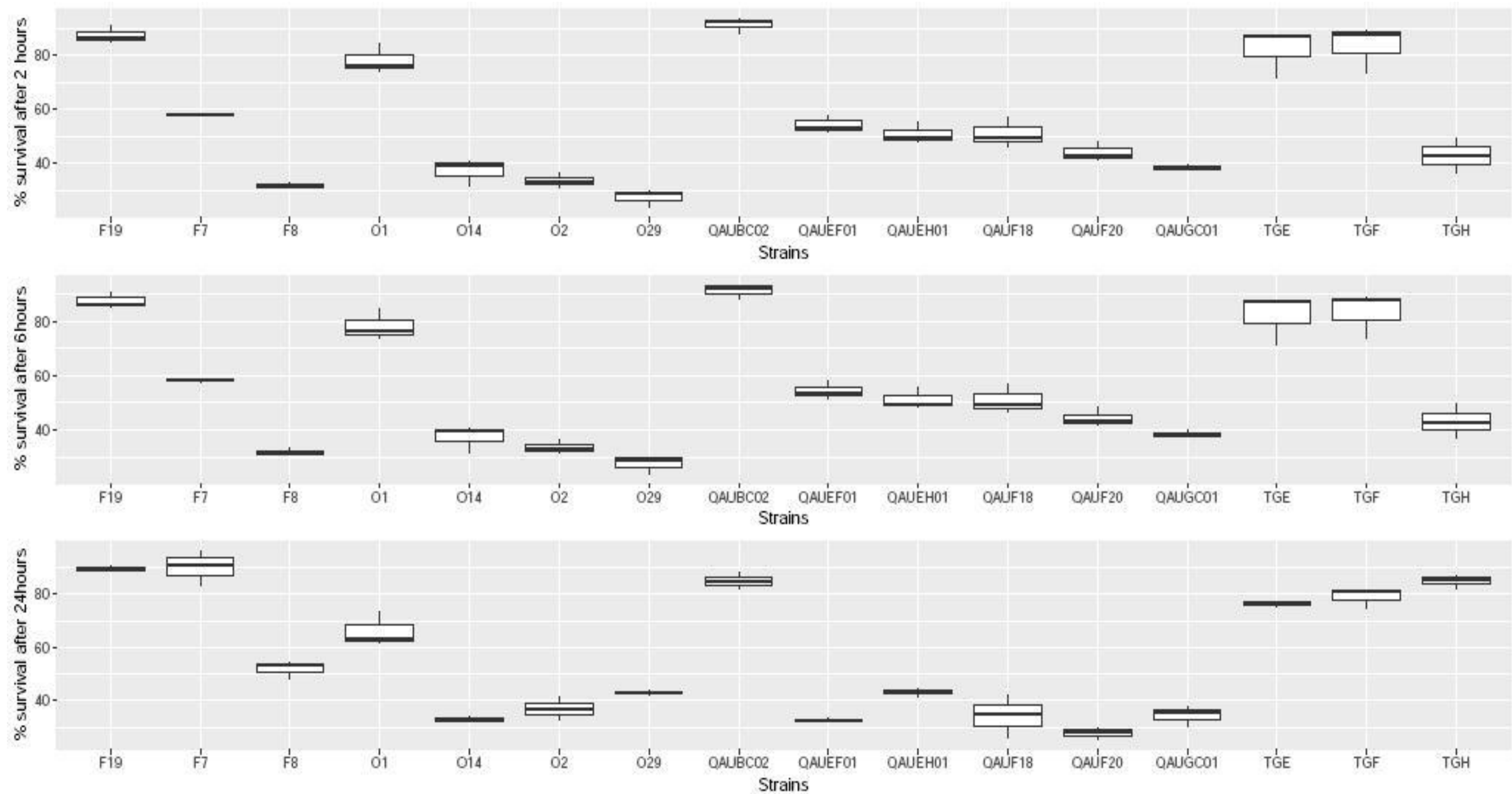


Figure 4. 7: **Percentage survival at different time intervals. This is the *box plot graphical respresentation of data generated in statistical R software.*.*QAUF20 (*E. mundtii* QAUF20),*QAUF18 (*E. faecium* QAUF18), ,*QAUBC02 (*B. cereus* QAUBC02), O1, O2, O29 , F7, F8, F19, O14 are unidentified fish gut isolates, TGF [*G. candidum* QAUGC01 and *B. cereus* QAUBC02], TGE(*G. candidum* QAUGC01 *and E. faecium* QAUEF01, TGH (*G. candidum* QAUGC01 and *E. hirae* QAUEH01), QAUGC01 (*G. candidum* QAUGC01), QAUEF01(*E. faecium* QAUEF01) , QAUEH01(*E. hirae* QAUEH01). This graph is the mean of three independent experiments**

4.3 Evaluation of hydrophobicity

The maximum hydrophobicity was showed by co-culture of *G. candidum* QAUGC01 in and *E. faecium* QAUEF01 (40.35±3.45), followed by *B. cereus* QAUBC02 (30.89±2.52) O2 (30.77±1.41), O14 (28.38±3.17), co-culture of *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (27.28±1.29), O1 (26.98±2.39), F8 (26.54±3.20), *E. faecium* QAUF18 (24.41±1.03), O29 (24.29±1.88), F19 (22.21±1.07), *G. candidum* QAUGC01 (21.96±1.78), F7 (18.81±0.88), *E. hirae* QAUEH01 (17.29±1.59), QAUEF01 (15.91±2.39), co-culture of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (11±1.74) and *E.mundtii* QAUF20 (5.14±1.57) as shown in Figure 4.8 and Table 4.2. The results are graphically represented by box plots, the height of box plot is related with the variation in the data.

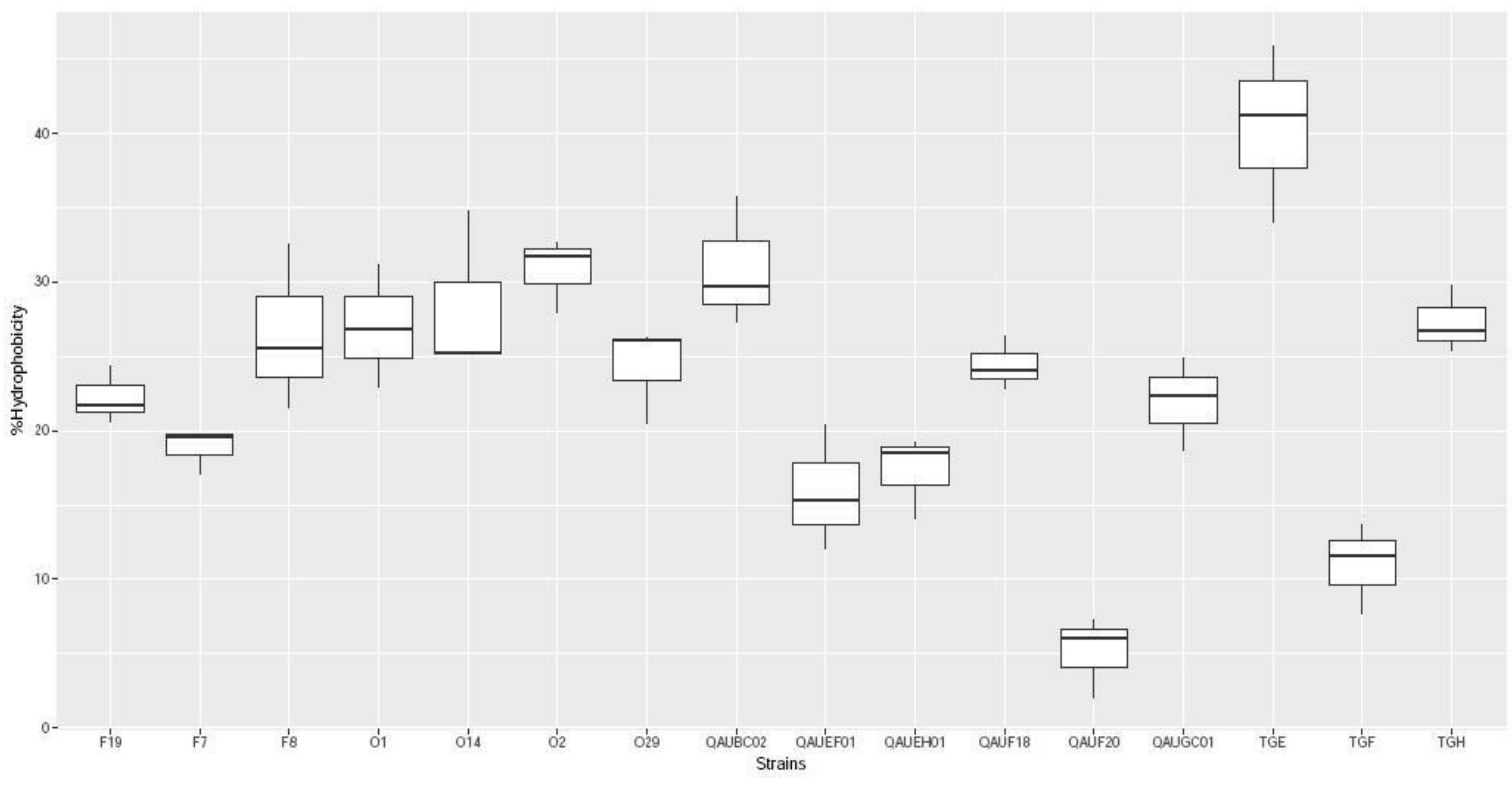


Figure 4. 8: **Percentage hydrophobicity of all strains. This is the box plot graphical respresentation of data generated in statistical R software..,*QAUF20 (*E. mundtii* QAUF20),*QAU F18 (*E. faecium* QAUF18), ,*QAUBC02 (*B. cereus* QAUBC02), O1 ,O2, O29, F7, F8, F19,O14 are unidentified fish gut isolates, TGF (*G. candidum* QAUGC01 and *B. cereus* QAUBC02), TGE (*G. candidum* QAUGC01 *and E. faecium* QAUEF01, TGH (*G. candidum* QAUGC0 1and *E. hirae* QAUEH01), QAUGC01 (*G. candidum* QAUGC01), QAUEF01 (*E. faecium* QAUEF01) , QAUEH01(*E. hirae* QAUEH01). This graph is the mean of three independent experiments.**

Table 4. 2: **Cumulative table showing invitro characterization of strains (single/ coculture) as potential probiotic candidates**

Sr. no.	Strains (single/coculture)	Percentage Hydrophobicity	Growth at pH 2	Growth at pH 5	Growth at pH 7	Growth at pH 9	% survival after 2 hours	% survival after 6 hours	% survival after 24 hours
1	***E. faecium*** **QAUEF01**	**15.91^{cd}(±2.40)**	**0.53^{a}(±0.005)**	**0.93^{ab}(±0.003)**	**1.49^{e}(±0.011)**	**0.75^{d} (±0.02)**	**54.36^{cd}(±1.94)**	**54.23^{c}(±4.95)**	**32.83^{ef}(±0.28)**
2	***E. hirae*** **QAUEH01**	**17.29^{cd}(±1.59)**	**0.35^{g}(±0.001)**	**0.73^{d}(±0.003)**	**1.73^{c}(±0.007)**	**0.86^{c}(±0.005)**	**51.16^{cde}(±2.21)**	**49.05^{c}(±1.39)**	**43.25^{de}(±0.92)**
3	***B. cereus*** **QAUBC02**	**30.89^{b}(±2.52)**	**0.15^{i}(±0.001)**	**0.35^{g}(±0.002)**	**0.16^{l}(±0.0020**	**0.25^{h}(±0.08)**	**91.47^{a}(±1.61)**	**87.31^{a}(±1.27)**	**85.01^{ab}(±1.85)**
4	***E. faecium*** **QAUF18**	**24.4^{bc}(±1.03)**	**0.42^{e} (±0.001)**	**0.84^{c}(±0.01)**	**1.35^{g}(±0.01)**	**0.53^{f}(±0.01)**	**51.06^{cde}(±3.16)**	**36.52^{cd}(±2.47)**	**34.43^{ef}(±4.57)**
5	**F19**	**22.2^{bc}(±1.07)**	**0.45^{cd}(±0.0036)**	**0.74^{d}(±0.005)**	**1.57^{d}(±0.006)**	**1.16^{b}(±0.02)**	**87.64^{a}(±1.76)**	**43.13^{a}(±3.05)**	**89.58^{a} (±0.42)**
6	***E. mundtii*** **QAUF20**	**5.14^{e}(±1.57)**	**0.38^{f} (±0.006)**	**0.95^{a}(±0.007)**	**1.406^{f}(±0.01)**	**0.82^{c}(±0.009)**	**44.36^{def}(±2.02)**	**35.60^{d}(±3.21)**	**27.81^{f}(±1.39)**
7	**F7**	**18.81^{cd}(±0.88)**	**0.47^{c}(±0.009)**	**0.88^{bc}(±0.007)**	**1.502^{e}(±0.004)**	**0.61^{e}(±0.011)**	**58.21^{bc}(±0.32)**	**66.09^{ab}(±3.15)**	**89.98^{a}(±3.72)**
8	**F8**	**26.54^{bc}(±3.20)**	**0.22^{i}±(0.002)**	**0.65^{e}(±0.004)**	**1.84^{a} (±0.002)**	**0.59^{e}(±0.01)**	**31.82^{fg}(±0.62)**	**73.25^{b}(±1.60)**	**51.90^{d}(±1.77)**
9	***G. candidum*** **QAUGC01**	**21.96^{bc}(±1.78)**	**0.06^{m}(±0.000)**	**0.21^{hi}(±0.003)**	**0.09^{l}(±0.00)**	**0.28^{h}(±0.04)**	**38.66^{efg}(±0.57)**	**33.39^{cd}(±1.62)**	**34.73^{ef}(±2.18)**
10	**O1**	**26.98^{bc}(±2.39)**	**0.073±(0.00)**	**0.702^{de}(±0.01)**	**1.77^{b}(±0.00)**	**0.34^{g}(±0.02)**	**78.21^{a}(±3.41)**	**95.31^{a}(±6.53)**	**66.09^{c}(±3.66)**
11	**O14**	**28.38^{bc}(±3.17)**	**0.521^{b}±(0.005)**	**0.86^{c}(±0.003)**	**1.32^{g}(±0.006)**	**1.31^{a}(±0.008)**	**37.43^{fg}(±2.84)**	**35.60^{cd}(±0.48)**	**33.04^{ef}(±0.54)**
12	**O2**	**30.77^{b}(±1.41)**	**0.447^{de} (±0.003)**	**0.47^{f}(±0.006)**	**1.05^{h}(±0.00)**	**0.37^{g}(±0.01)**	**33.59^{fg}(±1.51)**	**73.25^{b}(±3.36)**	**37.05^{ef}(±2.54)**
13	**O29**	**24.29^{bc}(±1.88)**	**0.42^{e} (±0.004)**	**0.032^{k}(±0.00)**	**0.26^{i} (±0.002)**	**0.11^{j}(±0.06)**	**27.49^{g}(±1.95)**	**66.28^{ab}(±4.09)**	**42.97^{de}(±0.53)**
14	**Co-culture of *G. candidum* QAUGC01 and *E. faecium* QAUEF01**	**40.35^{a}(±3.45)**	**0.134^{ij}(±0.24)**	**0.25^{h}±(0.025)**	**0.13^{jk}(±0.004)**	**0.75^{i}(±0.02)**	**82.31^{a}(±5.36)**	**69.34^{ab}(±2.98)**	**76.38^{bc}(±0.66)**
15	**Co-culture of *G. candidum* QAUGC01and *B. cereus* QAUBC02**	**11.00^{de}(±1.74)**	**0.101^{j}(±0.01)**	**0.14^{hi}(±0.03)**	**0.13^{jk}(±0.001)**	**0.20^{i}(±0.36)**	**83.59^{a}(±4.97)**	**88.21^{a}(±1.49)**	**79.10^{abc}(±2.13)**
16	**Co-culture of *G. candidum* QAUGC01and *E. hirae* QAUEH01**	**27.28^{bc}(±1.29)**	**0.24^{h}(±0.01)**	**0.17^{hi}(±0.001)**	**0.28^{i}(±0.005)**	**0.6^{e}(±0.01)**	**43.12^{def}(±3.67)**	**68.66^{ab}(±1.21)**	**84.82^{ab}(±1.42)**

*** *E. faecium,* QAUEF01, *E. hirae*, QAUEH01, *B. cereus* QAUBC02, *E. faecium* QAUF18, *E. mundtii* QAUF20, *G. candidum* QAUGC01, O1, O2, O29, F7, F8, F19, O14 are unidentified fish gut isolates, co-culture of *G. candidum* QAUGC01 *and E. faecium* QAUEF01, Co-culture *G. candidum* QAUGC01 and *B. cereus* QAUBC02, Co-culture of *G. candidum* QAUGC01 and *E. hirae* QAUEH01.**

4.4.4 Antibiotic susceptibility of bacterial strains

All the tested isolates were sensitive against Vancomycin (VA30), and Chloramphenicol (C 30). The strains that were resistant against Gentamycin (CN10) were F7, O29, *E. hirae* QAUEH01,O1. Intermediate zone was produced by F19, F8, *E. faecium* QAUF18. Sensitive strains were *E. mundtii* QAUF20, *E. faecium* QAUEF01, O14 and *B. cereus* QAUBC02. No zone was recorded against Cefpirome (CPO30), Ampicillin (AMP25), Ceftazidime (CAZ30), and Piperacillin (pr1100) by all tested strains. *B. cereus* QAUBC02 was sensitive against Ceftriaxone (CRO30). F7, F8, O29, *E. hirae* QAUEH01 and O1 produced intermediate zones against Ciprofloxacin (CIP5), *B. cereus* QAUBC02 was sensitive. O29, O14, O1 and *E. faecium* QAUEF01 were sensitive against Moxifloxacin (MXF5), *E. hirae* QAUEH01, F08, *E. faecium* QAUF18, *E. mundtii* QAUF20 and *B. cereus* QAUBC02 produced intermediate zones. The results are shown in table 4.3

Table 4. 3: Antibiotic sensitivity pattern of selected bacterial strains.

Antibiotics	F7	F8	O1	O14	O29	*E.hirae* QAUEH01	*E. faecium* QAUF18	*E.mundtii* QAUF20	*E. faecium* QAUEF01	*B. cereus* QAUBC02
Vancomycin	S	S	S	S	S	S	S	S	S	S
Cefpirome	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R
Gentamycin	R	I	R	S	R	I	I	S	S	S
Ceftriaxone	R	R	R	R	R	R	R	R	R	S
Ciprofloxacin	I	I	I	R	I	I	R	R	R	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S
Ceftazidime	R	R	R	R	R	R	R	R	R	S
Piperacillin	R	R	R	R	R	R	R	R	R	R
Moxifloxacin	R	I	S	S	S	R	I	I	S	I

*** *E. faecium* QAUEF01 (Dahi), *E. hirae* QAUEH01, *B. cereus* QAUBC02 , F7, F8, O1, O14, and O29 are unidentified fish isolates, *E. faecium* QAUF18 (Fish gut), and *Enterococcus mundti* QAUF20 (Fish gut).**

**** S :Sensitive I:Intermediate R: Resistatant**

***CLSI(Clinical and laboratory standards institute) guide lines were used for antibiotic susceptibity testing.**

4.4.5 Anti-Pathogenic activity

G. candidum QAUGC01 was effective against all the pathogens tested. Maximum activity against *L.* monocytogenes was recorded by co-culture of *G. candidum* QAUGC01 and *E. hirae* QAUEH01(28.5mm), *B. cereus* QAUBC02 was also active against *Listeria* producing a zone of 28mm. Maximum activity against *Staphylococcus aureus* was produced by *G. candidum* QAUGC0 (30.66mm). Maximum activity against *Pseudomonas aeruginosa* was recorded by co-culture of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (26.6mm). Results are shown in table 4.4.

Table 4. 4: Anti-pathogenic activity of the selected isolates and coculture

Isolates (Single/co-culture)	*P. aeruginosa* ATCC 27853	*S. enterica* ATCC14028	*E. coli* ATCC25922	*L. monocytogenes* ATCC49594	*S. aureus* ATCC 2593
F7	ND	ND	ND	ND	ND
F8	ND	ND	ND	ND	ND
O1	ND	ND	ND	18	16
O14	ND	ND	ND	21	13
O29	ND	ND	ND	26	13
***E. hirae* QAUEH01**	ND	ND	ND	22	25
***E. faecium* QAUF18**	ND	ND	ND	23	ND
***E. mundti* QAUF20**	ND	ND	ND	17	ND
***E. faecium* QAUEF01**	19	24	21	21	18
***B. cereus* QAUBC02**	ND*	ND	ND	28	20
***G. candidum* QAUGC01**	25.67	29	28.6	25.6	30.66
***E. faecium* QAUEF01**	19	24	21	21	18
***G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02 combination**	26.6	24.6	20.6	21.6	26.6
***G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01**	24.5	28	21	23.6	28.3
***G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01**	25	24.5	20	28.5	26

ND: not determined

** **O1, O29, O14, F7 and F8 are unidentified fish gut isolates**

4.5 Phase –II

Impact of selected probiotics on physiology of *Labeo rohita*

The second phase of the study comprised on probiotics used in single and co-culture form. The details are given in Table 4.5.

Table 4. 5: ***Probiotic supplemented treatments in 90 day feeding trial of* L. rohita *(10^9 CFU gm^{-1} diet).***

Groups	Probiotics lab codes	Strain name	NCBI accession number	Sourceof isolation
T0	Basal diet			
T1	QAUGC01	*Geotrichum candidum*	KT280407	Dahi
T2	QAUEF01	*Enterococcus faecium*	KP256006	Dahi
T3	QAUEH01	*Enterococcus hirae*	KP256015	Silage
T4	QAUBC02	*Bacillus cereus* (Fish gut isolated)	KT021872	Fish Gut
T5	QAUGC01 and QAUEF01(TGE)	Co-culture of *Geotrichum candidum* and *Enterococcus faecium*		
T6	QAUGC01 and QAUEH01(TGH)	Co-culture of *Geotrichum candidum* and *Enterococcus hirae*		
T7	QAUGC01 and QAUBC02(TGF)	Co-culture of *Geotrichum candidum* and fish gut isolated *Bacillus cereus*		
T8	Commercial probiotic	Consortia of Lactic acid bacteria and yeast		

4.5.1 Impact of probiotics on *L. rohita* Growth parameters.

4.5.1.1 Impact on weight gain

In response to the feed supplemented with T1= *G. candidum* QAUGC01, T2= *E. faecium* QAUEF01, T3= *E. hirae* QAUEH01, T4= *B. cereus* QAUBC02, T5= combination of *G. candidum* QAUGC01 and *E. faecium* QAUEF01, T6= combination of *G. candidum* QAUGC01 and *E. hirae* QAUEH01, T7= combination of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 and T8= commercial probiotic consortia, the effects on different growth parameters of fingerlings of *L. rohita* are presented in (Table 4.6). At the start of experiment

the initial body weights of all the treatments were measured and no significant difference (P<0.05) was found among all the treatments. After 45 day growth pattern showed that the weight gain (WG) of fish fed T7 (*G. candidum* QAUGC01and *B. cereus* QAUBC02 coculture) diet was significantly higher (P<0.05) than all other groups, while significantly lower WG was observed in group of fish fed basal diet (Table 4.6) same trend was shown by T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 coculture) 141.48 ±1.05 at 90th day significantly higher (P<0.05) value while lowest result was obtained with basal diet T0 and T4 which vary non significantly (Figure 4.9). The final biomass and biomass gain at 45th day and 90th day of feeding trial is represented in Table 4.7.

4.5.1.2 Impact on feed conversion ratios and feed efficiency ratios.

All of the potential probiotics alone or in combination significantly improved the FCR and FCE at 45 day (Table 4.6). The FCR values in *L. rohita* in response to T1 (*G. candidum* QAUGC01,) T2 (*E. faecium* QAUEF01), T3 (*E. hirae* QAUEH01) and T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) diets were 2.37±0.19, 2.3±0.13, 2.56±0.10, 2.29±0.07 respectively, which were significantly improved (P < 0.05) as compared to fish fed T4 (*B. cereus* QAUBC02)(3.27±0.25), T5 (*G. candidum* QAUGC01 and E. faecium QAUEF01) (2.63±0.11), T6 (*G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01) (3.36±0.14), T8 (Commercial probiotics) (2.97±0.07) and control diet (3.41±0.04). While significantly higher (P<0.05) FCE value was observed in T7(*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) (30.72±0.58). However, significantly lower FCE value was observed with basal diet (16.01±0.13) as compared to all other probiotic treatments. At 90days trial observations showed that the feeding of potential probiotics in single or co-culture form significantly improved the FCR and FCE.The highest FCR was 5.8279 ±0.15 in T4 fed with *B. cereus* QAUBC02, while lowest FCR % was noted in T2 (*E. faecium* QAUEF01) 5.02± 0.05. FCE % in T1, T2, T7 and T5 was 18.8204 ±0.22, 19.9220 ±0.199, 19.3866 ±0.23 and 18.1822 ±0.033 respectively. The FCE of T2 (*E. faecium* QAUEF01) and T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) was significantly higher than T0 (control), T4 (*B. cereus* QAUBC02), T8 (commercial probiotic) and T6 (*G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01) (Table 4.6 and Figure 4.10 and 4.11).

4.5.1.3 Impact of feeding treatments on specific growth rates of *L. rohita*

The specific growth rates (SGR) show a similar trend, significantly higher ($P<0.05$) value was observed with T7 diet both at 45day and 90 day while lowest result was obtained with basal diet.The specific growth rate was (2.67±0.04) at 45 for T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02) co-culture as compared to basal diet (1.29±0.07). T7 showed significantly higher value of specific growth rate (1.0931 ±0.05), while lowest result results was of T0 (0.7610 ±0.066) ($P<0.05$) at the 90 day of experiment (Figure 4.12). The final biomass at 45th day and 90th day is shown in table 4.7.

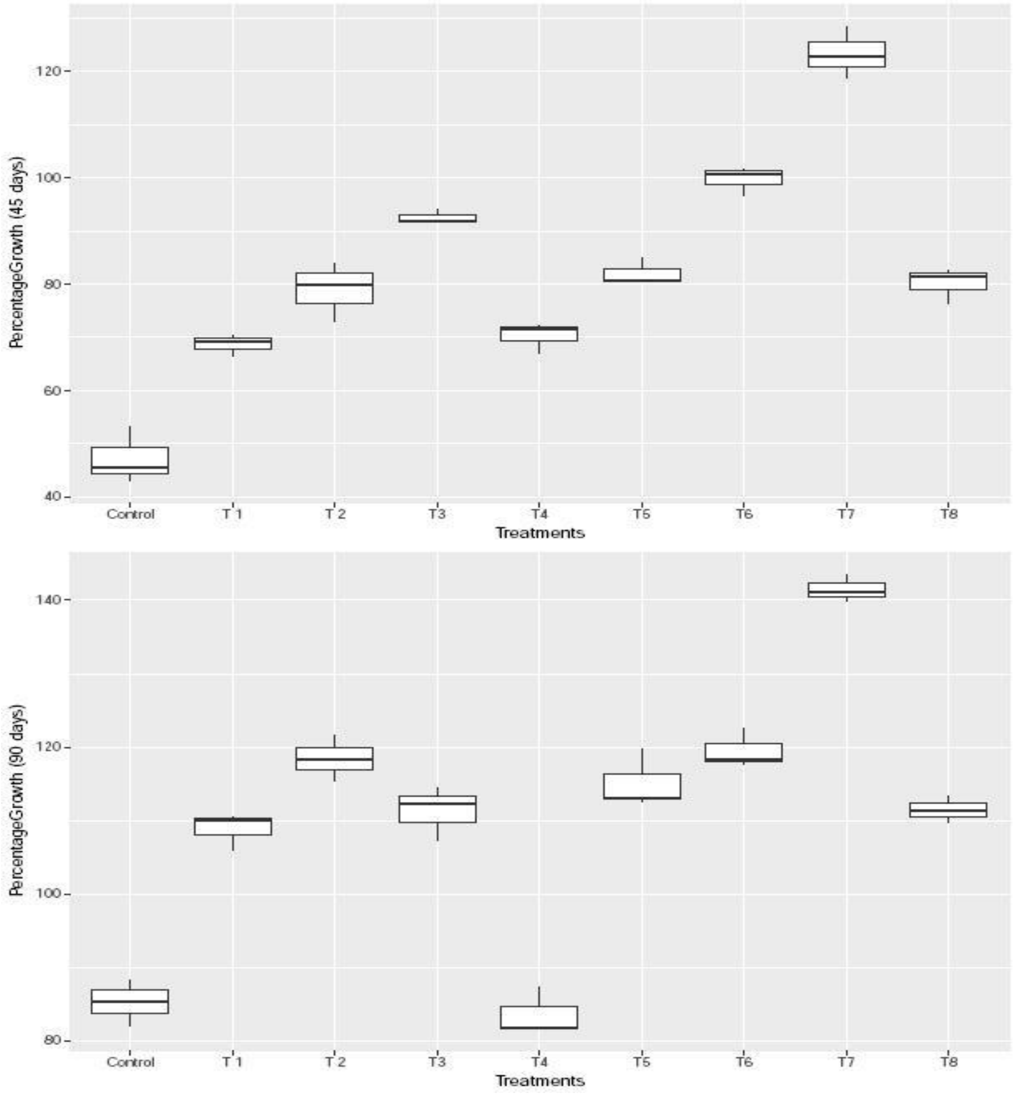


Figure 4. 9: Percentage growth of *L. rohita* after feeding for 45th and 90 th day on control (basal feed) and probiotics added treatments feed (T1-T8).This is the box plot graphical respresentation of data generated in R statistical software. *T1 (*G. n candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 coculture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 coculture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 coculture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).

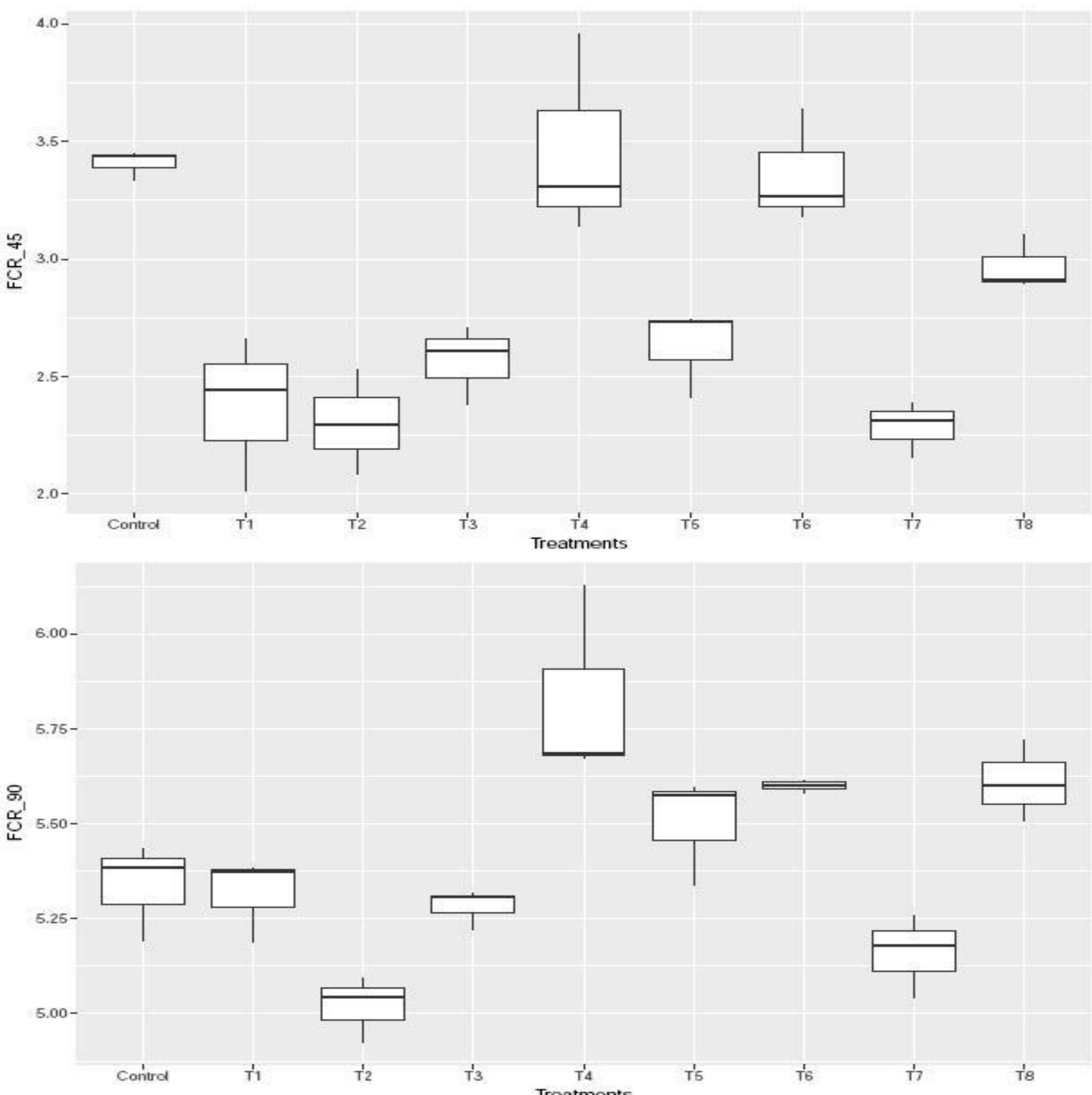


Figure 4. 10: Feed conversion ratio (FCR) of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software .

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

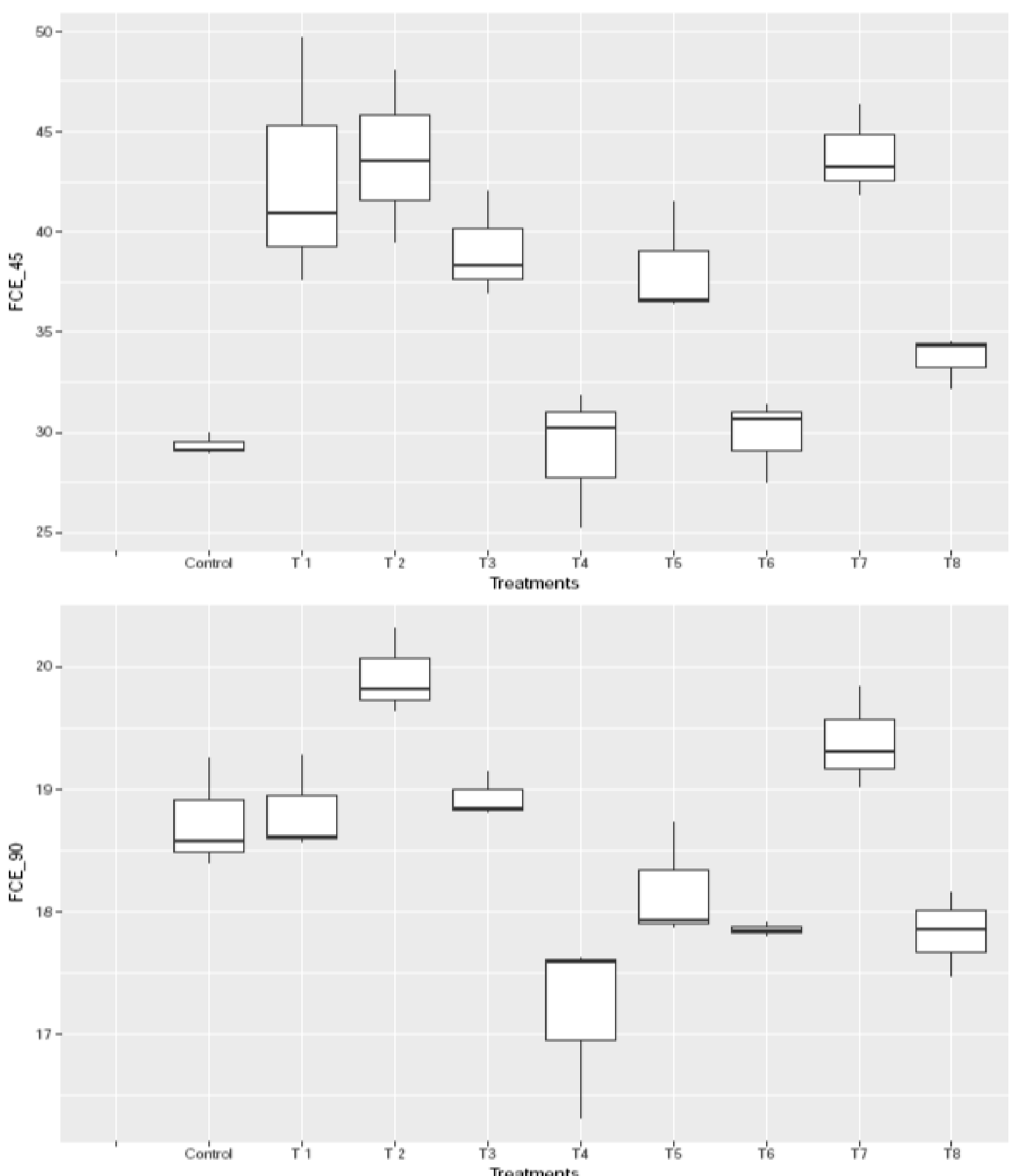


Figure 4. 11: Feed conversion efficiency (FCE) of *L. rohita* after feeding for 45th and 90 th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software .

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 coculture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 coculture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 coculture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

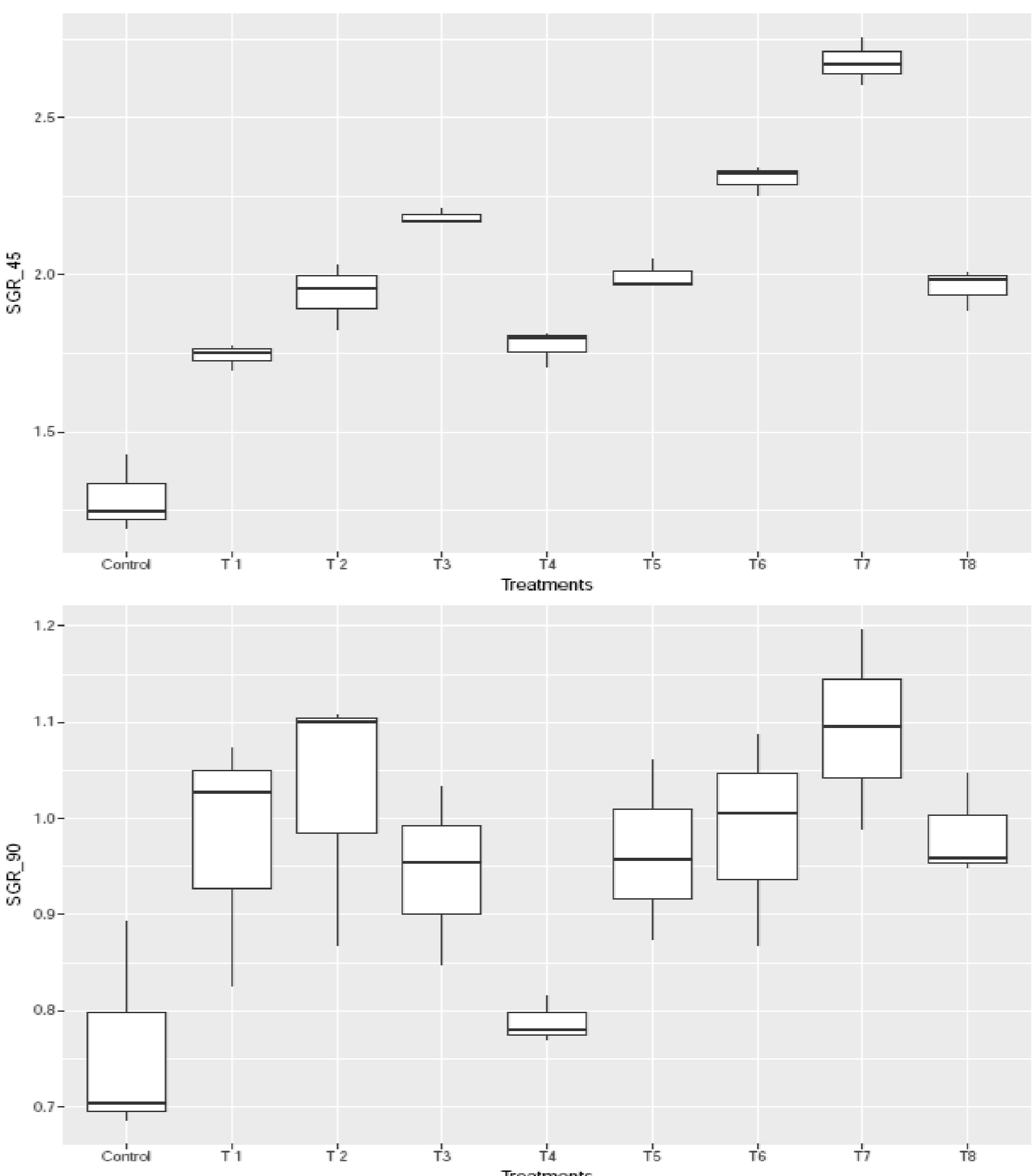


Figure 4. 12: Specific growth rate (SGR) of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software .

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

Table 4. 6: Effect of dietary administration of probiotic microorganisms (10^9 CFU gm^{-1} diet) in single and combined form, on growth performance of *L. rohita* fingerlings.

Groups	Weight Gain (%)WG		Specific Growth Rate (SGR)		Feed conversion Ratio (FCR)		Feed conversion Efficiency (%FCE)	
	45th day	90th day	45th day	90th day	45th day	90th day	45th day	90th day
T0	47.32 ^{f} (±3.09)	85.27 ^{e} (±1.80)	1.29 ^{f} (±0.07)	0.76 ^{c} (±0.06)	3.41 $^{a(}$ (±0.04)	5.3376 ^{cd} (±0.07)	16.01 ^{c} (±0.13)	18.74 ^{bc} (±0.26)
T1	68.69 ^{e} (±1.16)	108.08 ^{d} (±1.43)	1.74 ^{e} (±0.02)	0.97 ^{ab} (±0.075)	2.37 ^{d} (±0.19)	5.3149 ^{cd} (±0.06)	24.92 ^{a} (±1.46)	18.8204 ^{bc} (±0.229)
T2	78.98 ^{d} (±3.25)	118.43 ^{b} ±(1.82)	1.94 ^{d} (±0.06)	1.025 ^{a} (±0.078)	2.3 ^{d} (±0.13)	5.0206 ^{e} (±0.05)	26.35 ^{a} (±1.37)	19.9220 ^{a} (±0.34)
T3	92.55 ^{c} (±0.81)	111.34 ^{cd} (±2.16)	2.18 ^{c} (±0.01)	0.944 ^{ab} (±0. 053)	2.56 ^{cd} (±0.10)	5.2817 ^{bcd} (±0.03)	23.55 ^{ab} (±1.11)	18.9346 ^{bc} (±0.18)
T4	70.29 ^{e} (±1.68)	83.62 ^{e} (±1.88)	1.77 ^{e} (±0.03)	0.78 ^{bc} (±0.013)	3.27 ^{a} (±0.25)	5.8279 ^{a} (±0.15)	21.42 ^{c} (±1.47)	17.1806 ^{e} (±0.74)
T5	82.09 ^{d} (±1.37)	115.15 ^{bc} (±2.31)	1.99 ^{d} (±0.02)	1.97 ^{ab} (±0.05)	2.63 ^{cd} (±0.11)	5.5024 ^{bc} (±0.08)	28.06 ^{ab} (±0.62)	18.1822 ^{cd} (±0.47)
T6	99.71 ^{b} (±1.54)	119.51 ^{b} (±1.50)	2.3 ^{b} (±0.02)	0.098 ^{a} (±0.06)	3.36 ^{ab} (±0.14)	5.5993 ^{ab} (±0.01)	21.90 ^{c} (±0.91)	17.8593 ^{de} (±0.05)
T7	123.30 ^{a} (±2.79)	141.48 ^{a} (±1.05)	2.67 ^{a} (±0.04)	1.09 ^{a} (±0.059)	2.29 ^{d} (±0.07)	5.1598 ^{de} (±0.06)	30.72 ^{a} (±0.58)	19.3866 ^{ab} (± 0.41)
T8	80.07 ^{d} (±1.93)	111.49 ^{cd} (±1.03)	1.96 ^{d} (±0.03)	0.98 ^{a} (±0.03)	2.97 ^{bc} (±0.07)	5.6093 ^{ab} (±0.06)	23.89 ^{bc} (±0.44)	17.8321 ^{de} (±0.34)

* **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G.candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. ($P<0.05$) ANOVA followed by Duncan and Tukey analysis**

Table 4. 7: Effect of probiotic feeding (10^9 CFU gm^{-1} diet) in single and combined form on total biomass of *L. rohita* fingerlings after 45th and 90th days of feeding trial.

Groups	IBM**	FBM (45th day)	BMG (45th day)	FBM (90th day)	BMG (90th day)
T0	$61^{a(}$±0.51)	89.83^{a} (±1.17)	28.83^{f} (±1.64)	113.58^{f}(±0.58)	52^{f} (±0.76)
T1	61^{a}.00(±0.25)	102.90^{e} (±0.87)	41.90^{e}(±0.75)	127.4^{e}(±0.38)	66.40^{e} (±0.61)
T2	$60.27^{ab(}$±0.54)	107.83^{d} (±1.01)	47.57^{d}(±1.55)	131.62^{d} (±0.31)	71.36^{d} (±0.51)
T3	$60.33^{ab(}$±0.33)	116.17^{e}(±0.60)	55.83^{e}(±0.44)	127^{e}(±0.67)	67.17^{e} (±0.93)
T4	60.00^{ab}(±0.29)	102.17^{e}(±0.60)	42.17^{e}(±0.83)	110^{f}(±0.73)	50.17^{f} (±0.93)
T5	59.50^{b} (±0.46)	108.33^{d}(±0.44)	48.83^{d}(±0.52)	128^{d}(±0.76)	68.50^{d} (±0.96)
T6	60.33^{ab} (±0.33)	120.50^{b}(±1.44)	60.17^{b}(±1.17)	132.43^{b}(±0.74)	72.10^{b} (±0.74)
T7	61.00^{a}(±0.25)	136.20^{a}(±1.23)	75.20^{a}(±1.44)	147.30^{a}(±0.47)	86.30^{a} (±0.46)
T8	61.00^{a} (±0.50)	109.83^{d}(±0.94)	48.83^{d}(±0.98)	129^{d}(±0.58)	68^{d} (±0.29)

* **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G .candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis.**

****IBM initial body mass, FBM Final body mass, BMG body mass gain.**

4.6 Impact of probiotic treatment on hematological parameters

4.6.1 Hematology at 45th day of trial

The effect of dietary administration of probiotic microorganisms (10^9 CFU gm^{-1} of basal diet) in single and combined form on hematological parameters of *L. rohita* fingerlings (Table 4.8). According to the hematological parameters recorded at 45th day T2 (*E. faecium* QAUEF01) and T7(*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) fed groups of fishes had significantly higher (*$P<0.05$*) RBCs count (2.53±0.029) and

(2.43±0.02) respectively as compared to all other groups of fish. The control group of fish fed basal diet showed significantly lower level (1.57±0.017) of RBCs count as compared to groups of fish reared on single or combined probiotic supplemented feed (Table 4.8 and Figure 4.13). The same trend was seen in HGB (g dl^{-1}), MCH (pg), HCT% and MCHC (g dl^{-1}) values in different groups of *L. rohita.* Similarly T7(*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) showed significantly higher (P<0.05) HGB (g dl-1) concentration (9.7±0.35) in comparison to all other probiotic groups. Highest MCV was shown by T4 (139.66±0.88). Maximum platelet count was observed for T8 (77±0.57) and T6 (62±1.15) which was significantly higher than control groups.

4.6.2 Hematology at 90th day of trial

The impact of probiotic on hematology profile of 90th days presented in Table 4.8, which summarized the impact of feeding probiotics in single and co-culture form on hematological parameters such as RBCs, WBCs, HGB, MCH, MCHC, platelets and lymphocytes percentage in *L. rohita.* Fish fed with T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) , T6 (*G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01), T4 (*B. cereus* QAUBC02) and T8 (commercial probiotic) showed higher RBCs count and HGB level which was significantly (P<0.05) higher than T0 fed with basal diet. Highest RBCs count in T7 (co-culture of *G.candidum* QAUGC01 and *B. cereus* QAUBC02) was 2.7533±0.008 while lowest in T0 (basal diet) was 1.0667±0.008. Same trend was observed in HGB, T7 (co-culture of *G.candidum* QAUGC01 and *B. cereus* QAUBC02) 11.533±0.29 which was significantly (P<0.05) higher than T0 (control) 4.6000±0.34. HCT count of groups fed with probiotics was significantly higher than fish fed with basal diet. Highest HCT value 39.1333±0.72 was noted in T7 (co-culture of *G.candidum* QAUGC01 and *B. cereus* QAUBC02) and lowest 13.2667± 0.69 in T0 (control). HCT values of T1 (*G. candidum* QAUGC01), T5 (*E. faecium* QAUEF01), T6 (*G. candidum* QAUGC01 and *E.hirae* QAUEH01) and T8 (commercial probiotic) were also lower than T7 (co-culture of *G.candidum* QAUGC01 and *B. cereus* QAUBC02) and T4 group (*B. cereus* QAUBC02).

There was no significant difference between groups fed with probiotics supplemented diets and basal diet, but the highest MCH value 42.8000±0.838 was observed in T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01) group. Highest MCHC level in T6 (*G.*

candidum QAUGC01 and *E. hirae* QAUEH01) group was (64.9333±0.93) then second highest in T8 was (51.7000±0.56) that was significantly different from all other groups as well as T0 group fed on basal diets. Highest MCV was observed by T7 (143.53±0.87). There was significant variance in T7 (co-culture of *G.candidum* QAUGC01 and *B. cereus* QAUBC02) group fed with among all other groups in RBC, HGB and HCT level. T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01) fed with and T6 (*G.candidum* QAUGC01 co-culture with *E. hirae* QAUEH01) fed with showed significant variance among all groups in MCH and MCHC respectively (Figure 4.13, 4.14, 4.15.4.16.4.17 and 4.18 Table 4.8).

Figure 4. 13: Red Blood Cells (RBC) counts of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

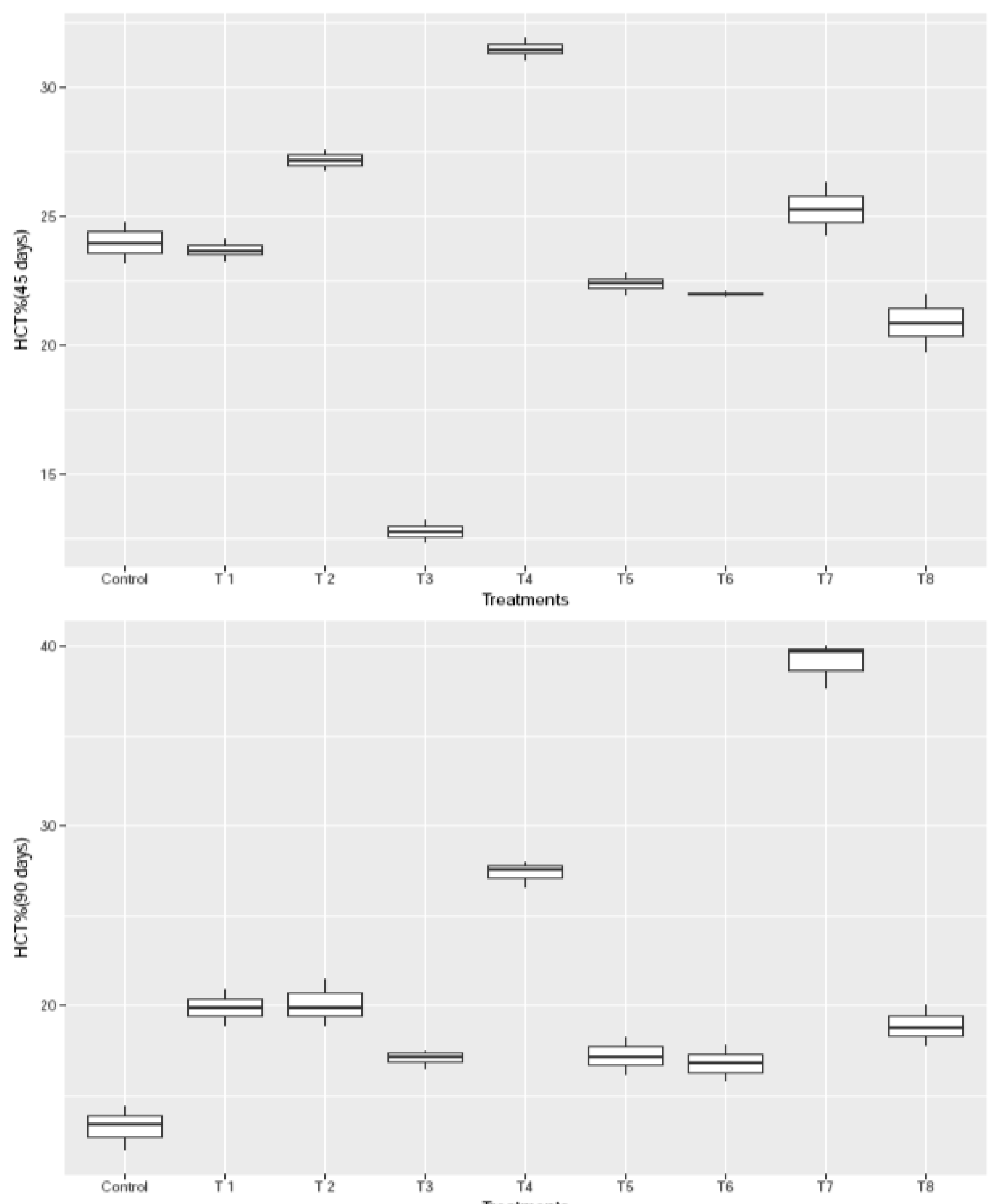


Figure 4. 14: Hematocrit (HCT) percentage of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

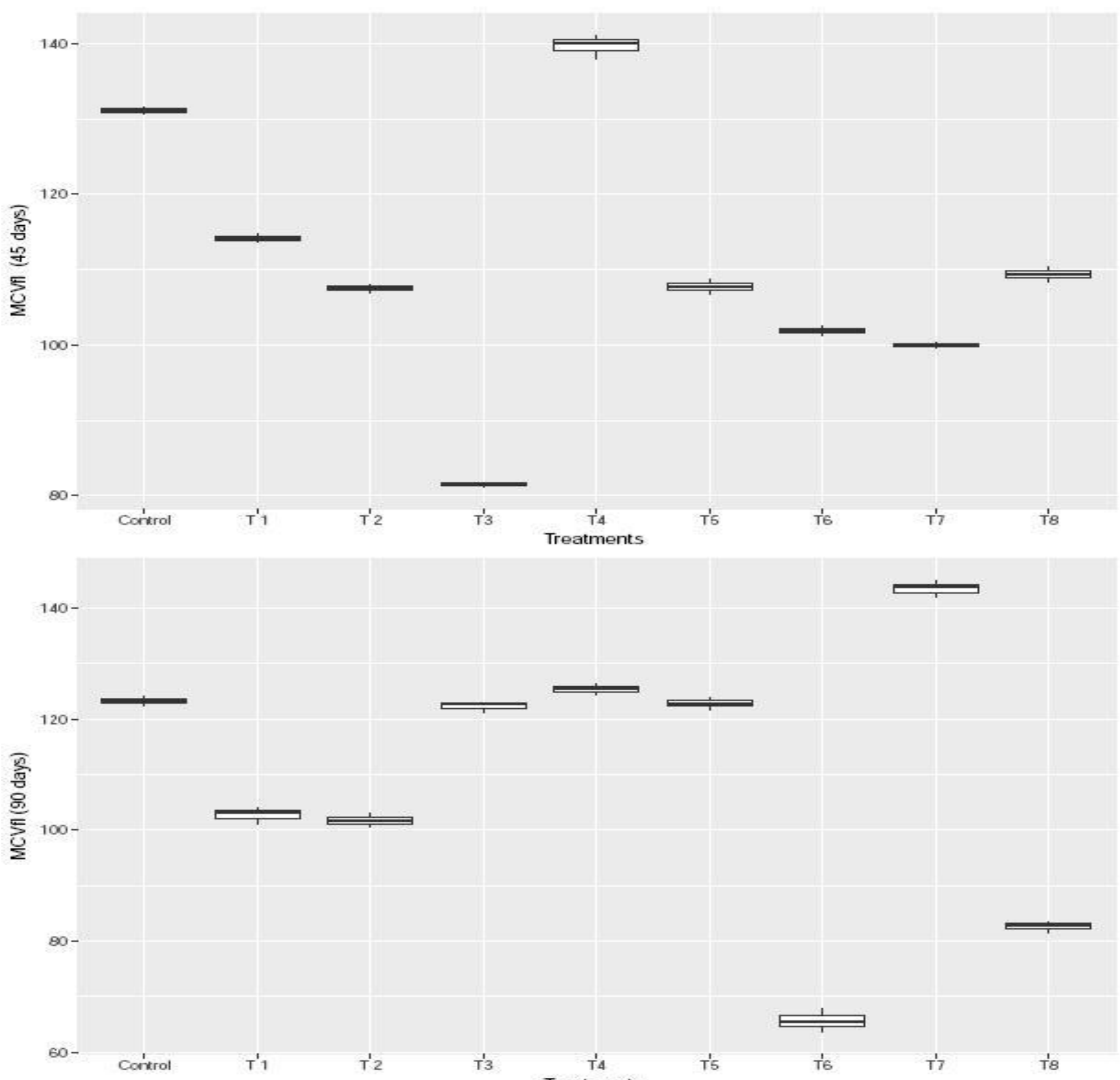


Figure 4. 15: Mean corpuscular volume (MCV) of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

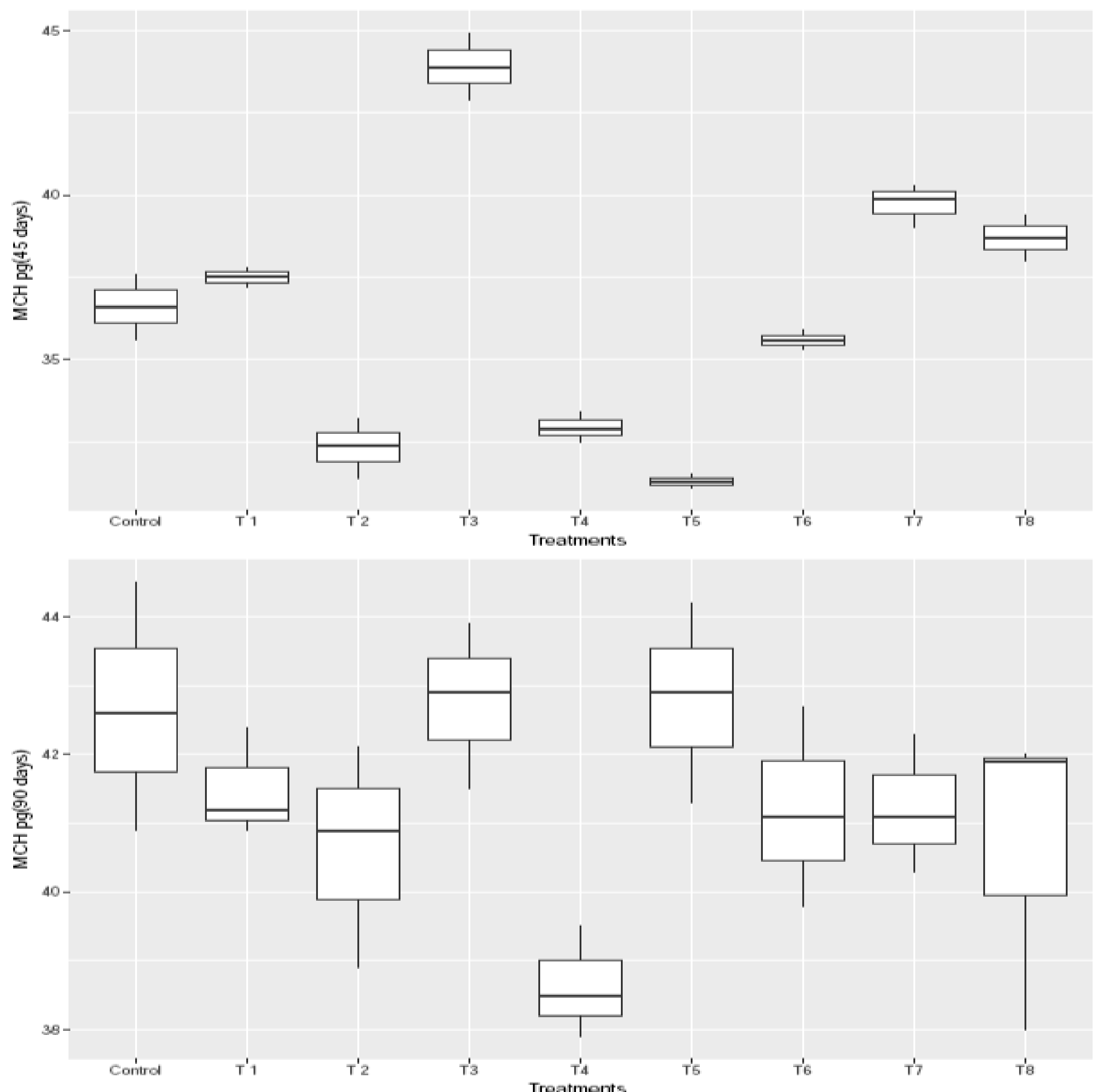


Figure 4. 16: Mean corpuscular hemoglobin (MCH) concentration of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

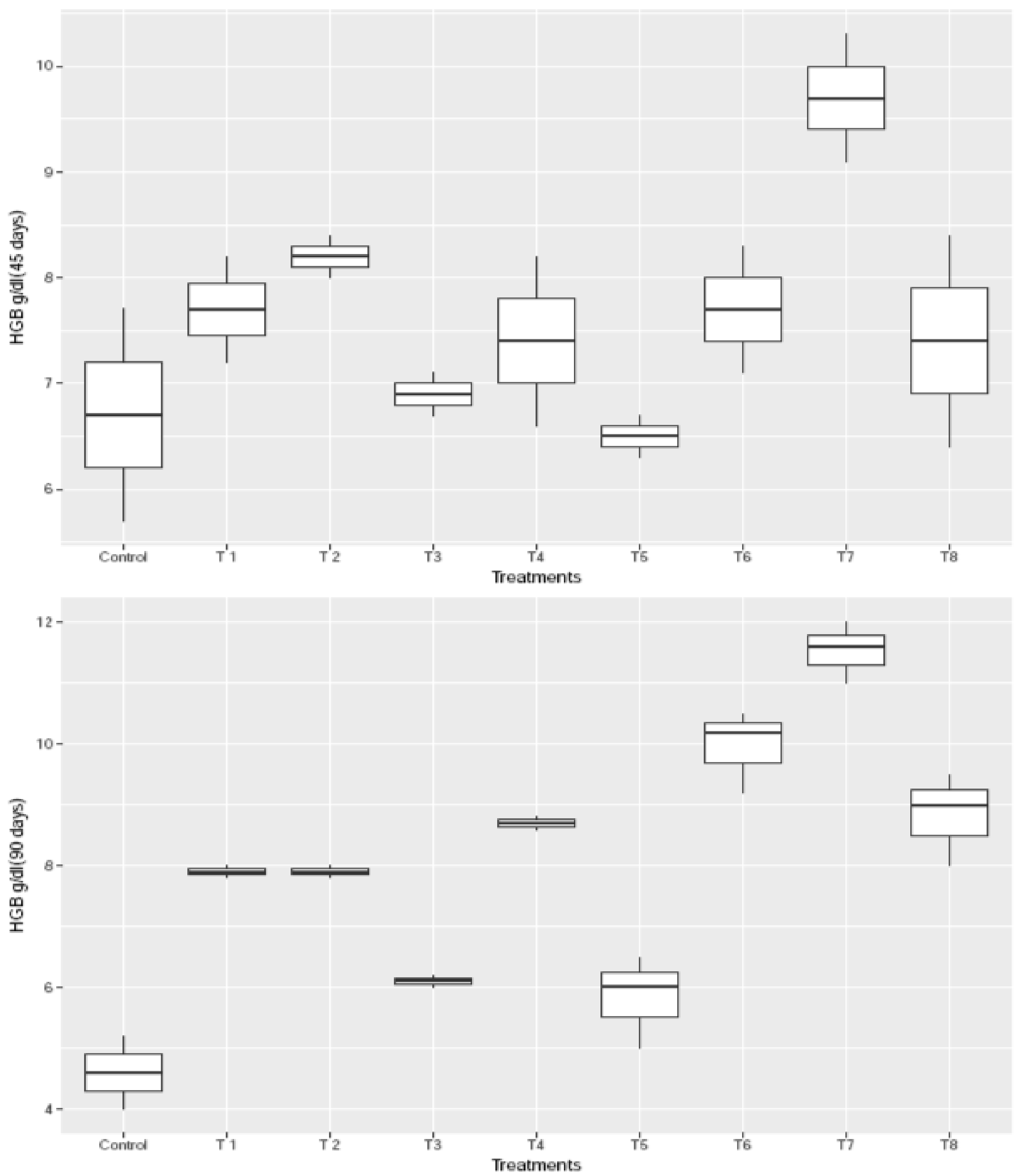


Figure 4. 17: Hemoglobin (HGB) concentration of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

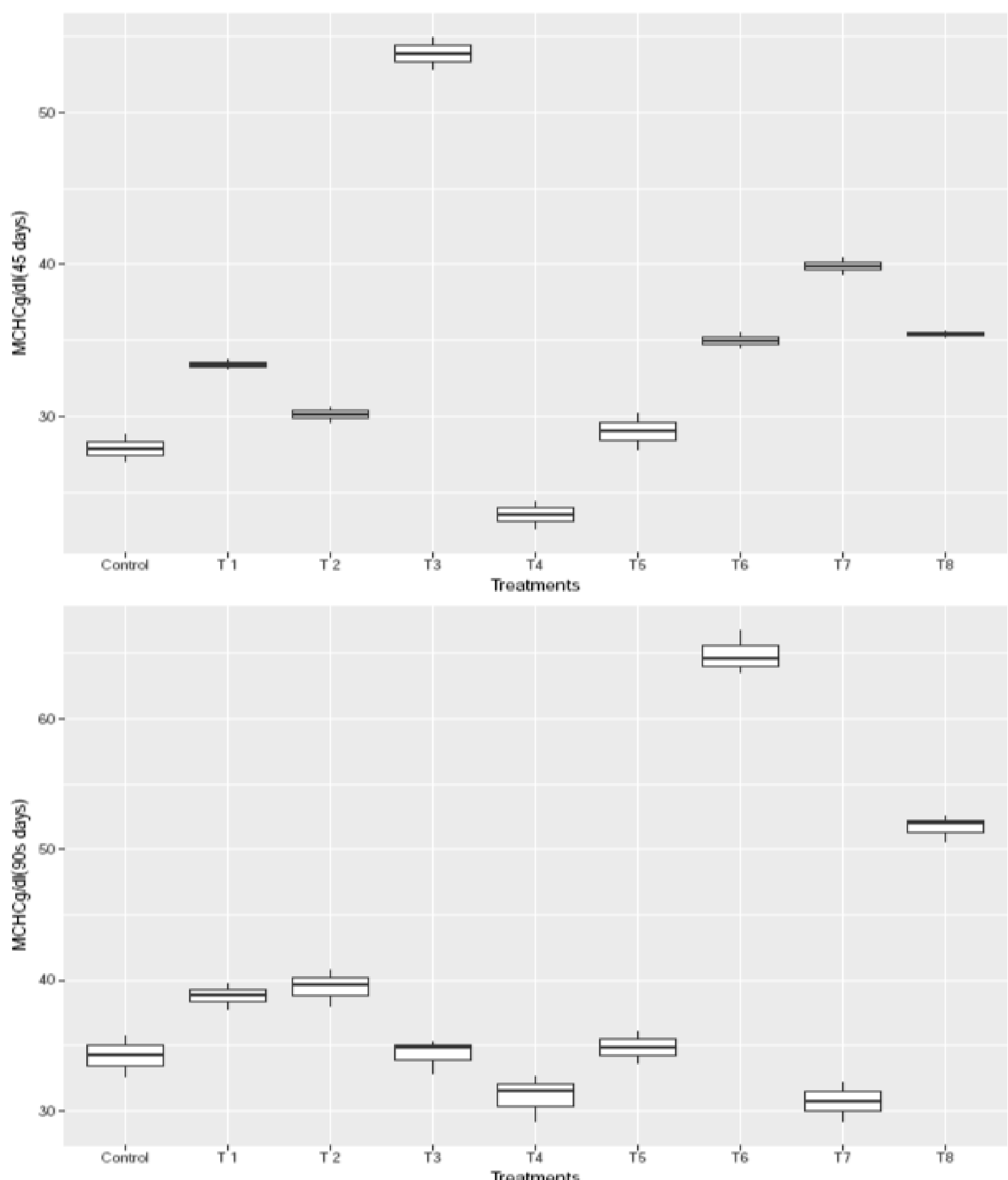


Figure 4. 18: Mean corpuscular hemoglobin concentration (MCHC) of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9)**

Table 4. 8: Cummulative data of hematological parameters of feeding trials of *L. rohita*

Groups	Red blood cells RBCs ($10^6 \mu L^{-1}$)		Hemoglobin HGB ($g\ dl^{-1}$)		Hematocrit HCT (%)		Mean corpuscular hemoglobin MCH (pg)		Mean corpuscular hemoglobin concentration MCHC ($g\ dl^{-1}$)		Mean corpuscular volumes MCVfl	
	45th day	90th day	45th day	90th day	45th day	90th day	45th day	90th day	45th day	90th day	45th day	90th day
T0	1.57 ^{d} (±0.017)	1.0667 ^{G} (±0.01)	6.7 ^{b} (±0.58)	4.600 ^{e} (±0.34)	24 ^{cd} (±0.46)	13.266 ^{f} (±0.69)	36.6 ^{d} (±0.58)	42.6667 a (±1.03)	27.9 ^{e} (±0.52)	34.20 ^{de} ±(0.28)	131.1 ^{b} (±0.28)	40.66 ^{b} (±1.06)
T1	2.21 ^{b} (±0.061)	1.9600 $^{-d}$ (±0.02)	7.7 ^{ab} (±0.29)	7.900 ^{c} (±0.057)	23.7 ^{cde} (±0.23)	19.90 ^{cd} (±0.57)	37.5 ^{cd} (±0.17)	41.500 ^{a} (±0.45)	33.4 ^{c} (±0.17)	38.8 ^{c} (±0.55)	114.2 ^{c} (±0.28)	103.1 ^{c} (±0.90)
T2	2.53 ^{c} (±0.029)	1.9967 ^{cd} (±0.052)	8.2 ^{b} (±0.12)	7.900 ^{c} (±0.057)	27.2 ^{b} (±0.23)	20.10 ^{c} (±0.75)	32.33 ^{e} (±0.52)	40.6 ^{ab} (3±0.93)	30.1 ^{d} (±0.29)	39.4 ^{c} ± (0.78)	107.5 ^{d} (±0.34)	101.7 ^{c} (±0.75)
T3	1.83 ^{e} (±0.023)	1.4000 ^{e} (±0.057)	6.9 ^{b} (±0.12)	6.100 ^{d} (±0.057)	12.8 ^{g} (±0.23)	17.066 ^{de} (±0.29)	43.9 ^{a} (±0.58)	42.76 ^{a} (±0.69)	53.9 ^{a} (±0.58)	34.30 ^{de} (±0.75)	81.5 ^{f} (±0.17)	122.33 ^{b} (±0.61)
T4	2.25 ^{b} (±0.081)	2.2033 ^{c} (±0.054)	7.4 ^{b} (±0.46)	8.70 ^{bc} (±0.057)	31.5 ^{a} (±0.23)	27.400 ^{b} (±0.416)	32.93 ^{e} (±0.26)	38.63 ^{b} (±0.46)	23.5 ^{f} (±0.52)	31.10 ^{de} (±1.00)	139.66 ^{a} (±0.88)	125.43 ^{b} (±0.63)
T5	2.08 ^{c} (±0.023)	1.5667 ^{e} (±0.088)	6.5 ^{b} (±0.12)	5.83 ^{de} (±0.44)	22.4 ^{def} (±0.23)	17.200 ^{cde} (±0.57)	31.3 ^{e} (±0.12)	42.80 ^{a} (±0.83)	29 ^{de} (±0.69)	34.8 ^{d} (±0.69)	107.7 ^{d} (±0.57)	122.35 ^{b} (±0.606)
T6	2.16 $^{bc(}$ (±0.032)	2.4833 ^{b} (±0.008)	7.7 ^{b} (±0.35)	9.966 ^{b} (±0.39)	22 ^{ef} (±0.06)	16.80 ^{e} (±0.57)	35.6 ^{d} (±0.17)	41.20 ^{ab} 00.83	35 ^{c} (±0.29)	64.93 ^{a} (±0.93)	101.9 ^{e} (±0.34)	65.7 ^{e} (±1.18)
T7	2.43 ^{a} (±0.023)	2.7533 ^{a} (±0.008)	9.7 ^{a} (±0.35)	11.53 ^{a} (±0.29)	25.3 ^{c} (±0.58)	39.13 ^{a} (±0.72)	39.7367 ^{b} (±0. 12)	41.2 ^{ab} (±0.58)	39.9 ^{b} (±0.29)	30.7000± 0.86 ^{e}	100±0.17 ^{e}	143.53 ^{a} (±0.8)7
T8	1.91 ^{c} (±0.023)	2.1300 ^{c} (±0.008)	7.4 ^{b} (±0.58)	8.8333 ^{bc} (±0.44)	20.9 ^{f} (±0.64)	18.86 ^{cde} (±0.63)	0.38 ^{bc} (±0.40)	40.6 ^{ab} (±1.31)	35.4 ^{c} (±0.12	51.7000± 0.57 ^{b}	109.4±0.57 ^{d}	40.6 ^{s} (±1.31)

*** Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E.hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. ($P<0.05$) ANOVA followed by Duncan and Tukey analysis.**

4.7 Impact of probiotic feeding on Serum parameters (WBC, Platelets and Lymphocytes) of *L. rohita*.

45 days data showed that in *L. rohita* significantly higher (P<0.05) WBC (223.8±0.57) was observed in T7 treatment fed with (*G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02) as compared to all other treatments. The means of all the treatments vary significantly from the group fed on basal diet (178.4±0.57). Minimum WBC count after 45 was observed in T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) (159.2±0.28). 90 days of experimental trials showed that in *L. rohita* significantly higher (P<0.05) WBC count (253.6000±1.171) was observed in T7 (*G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02) group of fish fed with (*G.candidum* QAUGC01 and *B.cereus* QAUBC02 co-ulture), as compared to all other groups (Figure 4.19). However, group T6 (coculture of *G.candidum* QAUGC01 and *E.hirae* QAUEH01), T7 (*G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02) and T8 (commercial probiotic) had no significant difference in WBC count but they were significantly different (P<0.05) from T0 group (basal diet) 142.2667±1.21 which fed with basal diet. Platelets count at 45 day observation showed highest count in T8 (commercial probiotic) 252.3000±0.49, T2 (*E. faecium* QAUEF01) and T0 (basal diet) were not significantly (P<0.05) different from one another, similarly T4 (*B.cereus* QAUBC02) and T1 (*G. candidum* QAUGC01) were not significantly different from one another, lowest count was showed by T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01). Platelets count of all treated groups at 90 day was not significantly (P<0.05) different from the T0 control group fed with basal diet except T8 group which showed significant difference. T8 (commercial probiotic) showed highest platelets count among all groups (387.6667±2.51) while lower count was showed by the T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01), T4 (*B.cereus* QAUBC02), T3 (*E.hirae* QAUEH01), T7 (*G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02) and T0 (basal diet) (Figure 4.20). Lymphocyte percentage at 45 day of all the treatments showed no statistically significant difference between all the treatments regarding lymphocyte percentage at 45 day highest value given by T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) (98.9±0.057) and lowest was observed in T3 (*E. hirae* QAUEH01) (97.4±0.17). There was no statistically significant difference (P<0.05)

between T1(*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T4 (*B. cereus* QAUBC02), T6 (co-culture of *G.candidum* QAUGC01 and *E. hirae* QAUEH01), T7 (*G. candidum* QAUGC01 in combination with *B. cereus* QAUBC02) and T8 (commercial probiotics)for lymphocytes count, but these groups were significantly different from T0, T3(*E. hirae* QAUEH01) and T5(*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) group. Highest lymphocytes percentage at 90th day was observed in T4 (97.8667±0.95) and lowest was in T0 (77.6667±0.52) control group (Figure 4.21)(Table 4.9).

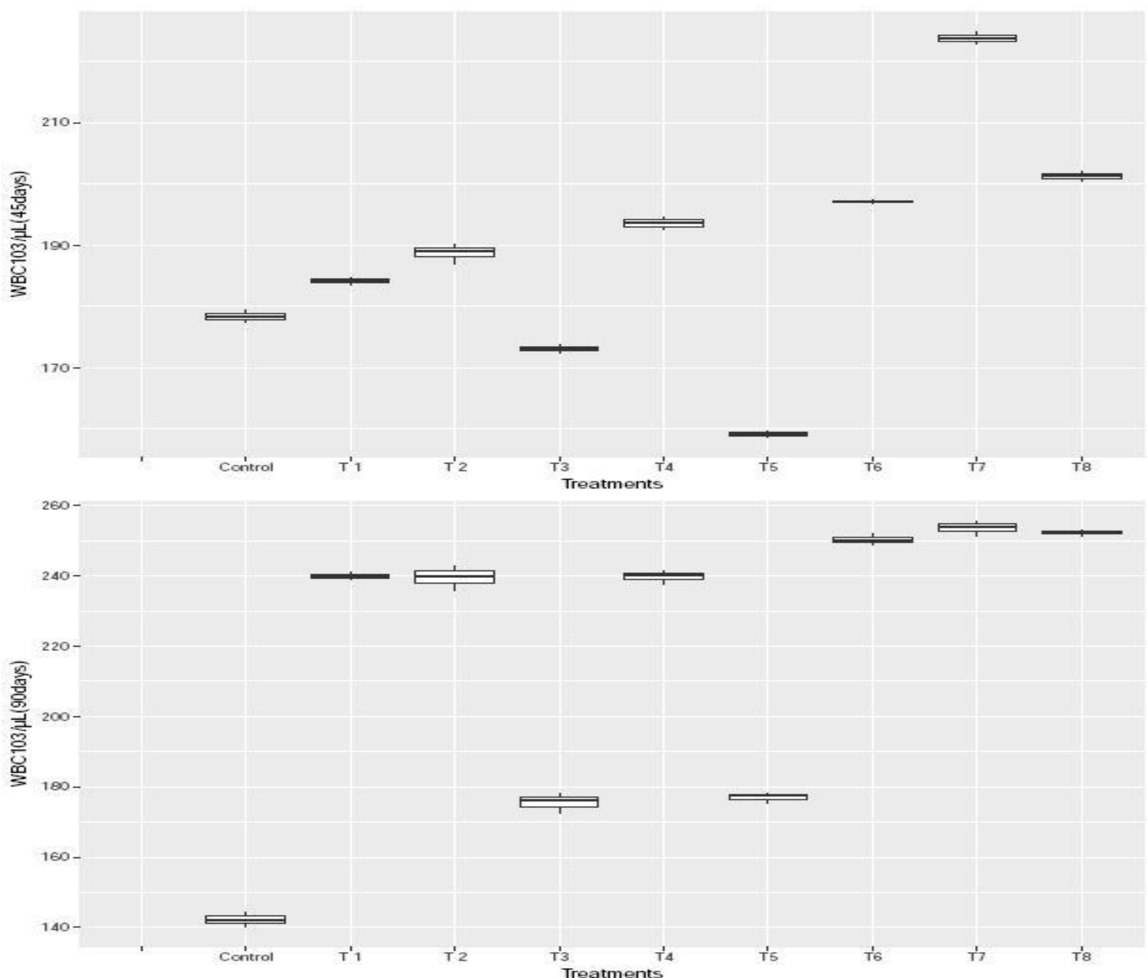


Figure 4. 19: White blood cells (WBC) counts of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

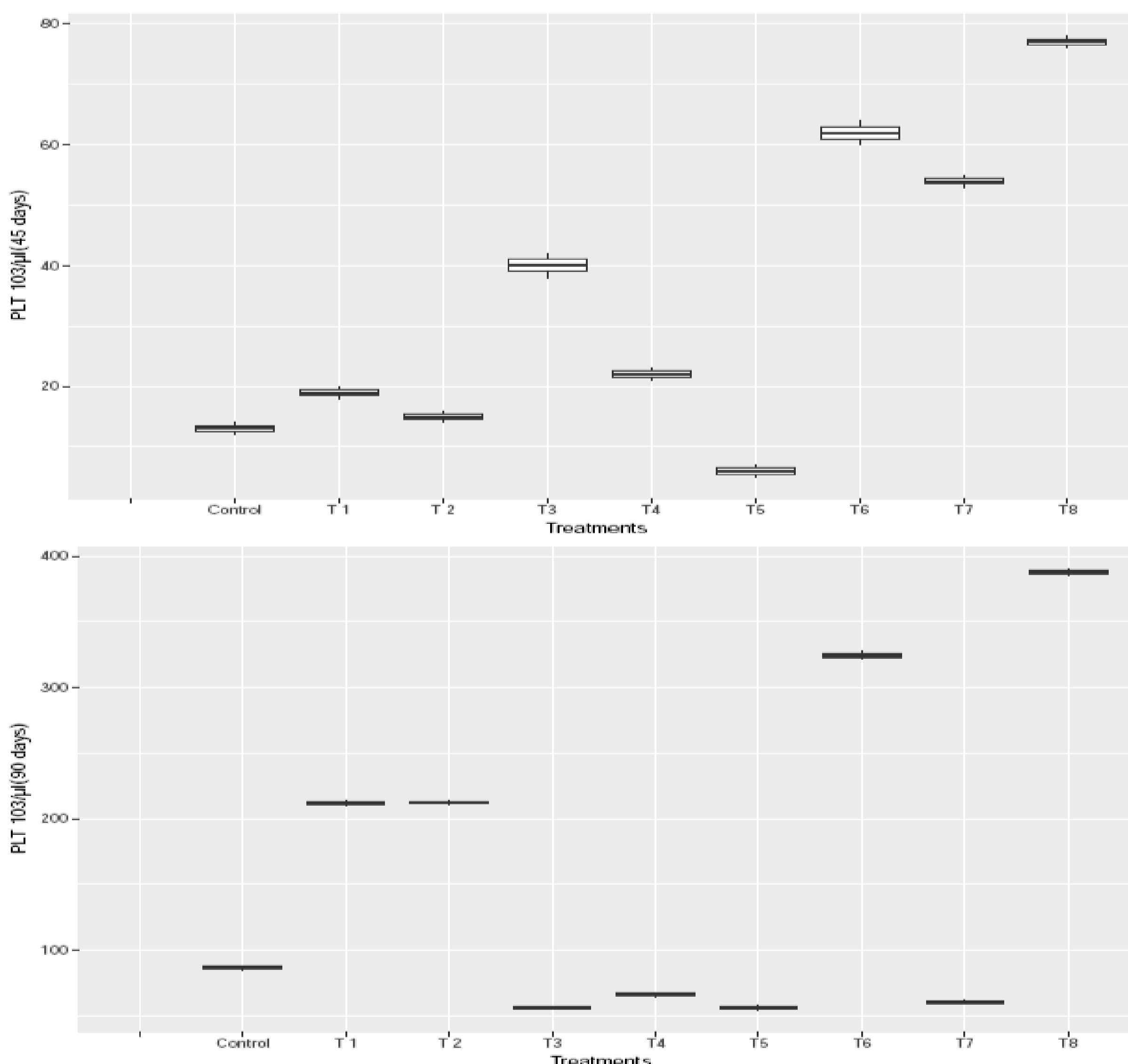


Figure 4. 20: Platelets (PLT) counts of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

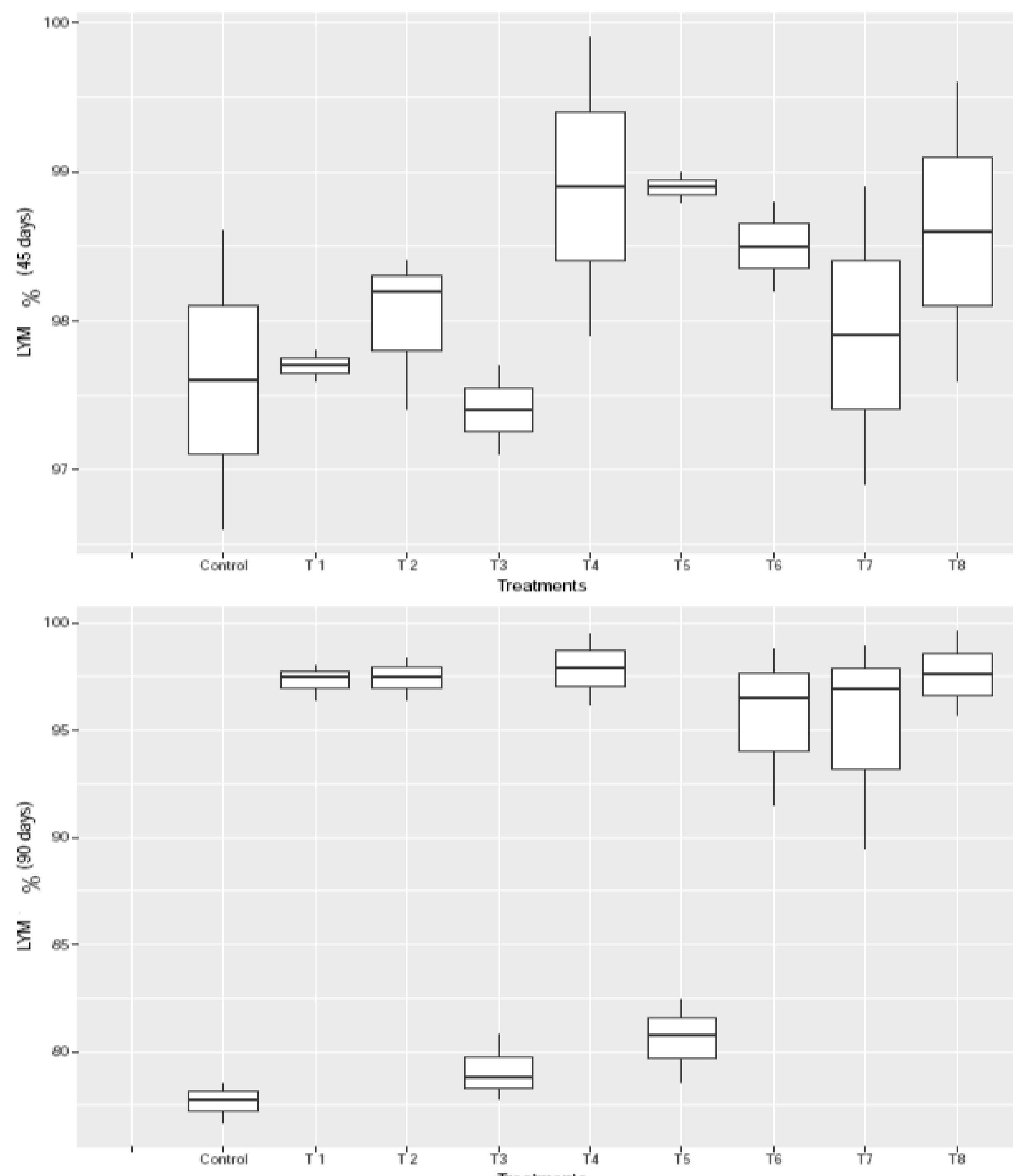


Figure 4. 21: Lymphocyte percentage (LYM) of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

Table 4. 9*:* Cummulative data for serum parameters (White blood cells count, platelets count and Lymphocyte (%)) of *L. rohita* impact of feeding probiotic microorganisms (10^9 CFU/gm diet) in single and coculture.

Groups	White blood cells WBC (10^3/µl)		Platelets PLT(10^3/µl)		Lymphocytes lym %	
	45th day	90th day	45th day	90th day	45th day	90th day
T0	178.4 ^{g} (±0.57)	142.26 ^{d} (±1.21)	13 ^{a} (±0.57)	86.6667 ^{d} (±0.88)	97.6 ^{a} (±0.57)	77.66 ^{b} (±0.52)
T1	184.2 ^{f}(±0.34)	240.0 ^{b} (±0.57)	19 ^{f} (±0.57)	212.00 ^{c} (±1.15)	97.7 ^{a} (±0.057)	97.30 ^{a} (±0.47)
T2	188.7 ^{e} (±0.93)	239.66 ^{b} (±2.02)	15 ^{e} (±0.57)	212.30 ^{c} (±0.68)	98 ^{a} (±0.30)	97.43 ^{a} (±0.57)
T3	173.1 ^{h} (±0.40)	175.56 ^{c} (±1.69)	40 ^{f} (±0.57)	55.90 ^{f} (±0.66)	97.4 ^{a} (±0.17)	79.133 ^{b} (±0.88)
T4	193.6 ^{d} (±0.63)	239.83 ^{b} (±1.121)	22^{b}(±0.57)	65.833 ^{e} (±0.92)	98.9 ^{a} (±0.57)	97.86 ^{a} (±0.95)
T5	159.2 ^{i} (±0.28)	177.0333 ^{c} (±2.48)	6.0 ^{e} (±0.57)	55.9333 ^{f} (±1.09)	98.9 ^{a} (±0.057)	80.60 ^{b} (±1.10)
T6	197.1 ^{c} (±0.97)	250.3333 ^{a} (±0.88)	62 ^{g} (±0.57)	324.666 ^{b} (±1.76)	98.5 ^{a} (±0.17)	95.6000 ^{a} (±2.15)
T7	223.8 ^{a} (±0.57)	253.600 ^{a} (±1.171)	54 ^{b} (±0.57)	60.333 ^{ef} (±1.05)	97.9 ^{a} (±0.57)	95.1000 ^{a} (±2.85)
T8	201.3 ^{b} (±0.46)	252.3000 ^{a} (±0.49)	77 ^{c}(±0.57)	387.666 ^{a} (±1.45)	98.6 ^{a} (±0.57)	97.6333 ^{a} (±1.12)

* **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G .candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis**

4.8 Impact of feeding treatments on intestinal enzymatic activity

Comparative specific activities of the intestinal enzymes of *L. rohita* fingerlings after 45th days of feeding trial on a basal, single strain, combine and commercial probiotic supplemented diet is shown in Table 4.10.

4.8.1 Evaluation of enzyme activity at 45th and 90th day

Intestinal enzyme activity of *L. rohita* fingerlings after 45 days trial of probiotics feeding in single and coculture is summarized in (Table 4.10). Significantly higher (P<0.05) value was observed in intestinal protease activity in group T5 (0.0263±0.00009), fed with *G. candidum* QAUGC01 and *E. faecium* QAUEF01 combined diet as compared to basal (0.0186±0.00008) fed diet. Probiotic group T2 (*E. faecium* QAUEF01) showed the least protease activity (0.0152±0.00015), however T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01), T6 (*G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01) and T7(*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) showed significantly higher (P<0.05) protease activity in comparison with control (C) i.e. (0.0263±0.00009), (0.0197±0.00002) and (0.0199±0.0001) respectively. Highest value of protease activity at 90th day was observed in T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01) group was (0.0262±0.00009) while lowest activity was in T1 (*G. candidum* QAUGC01) group (0.0138±0.000064) (Table 4.10, Fig4.22).

Intestinal enzymes activity of *L. rohita* after 90 days trial of probiotics feeding in single and consortium form is summarized in (Table 4.10). There was no statistically significant difference (P<0.05) between T0 control group fed with basal diet and all treated group fed with probiotics supplemented diet except T5 which was significantly different (P<0.05) from T0 and all other groups in protease activity.

Maximum Amylase activity at 45/90 day was observed for T4 (0.04±0.00001) which is significantly higher than control T0 (0.025±0.0001) while at 90 day of trial maximum amylase activity given by *B. cereus* QAUBC02was (0.0413±0.000054) which was significantly higher than control T0 (0.0320±0.0001) (Figure 4.23).

Maximum cellulase activity at 45/90 day trial was observed by T4 *B. cereus* QAUBC02 having values (0.214±0.004) at 45 day and (0.265±0.001) at 90 day which is significantly higher than control T0 (0.154±0.0009) at 45 day and (0.163±0.0005) at 90th day (Figure 4.24, Table 4.10).

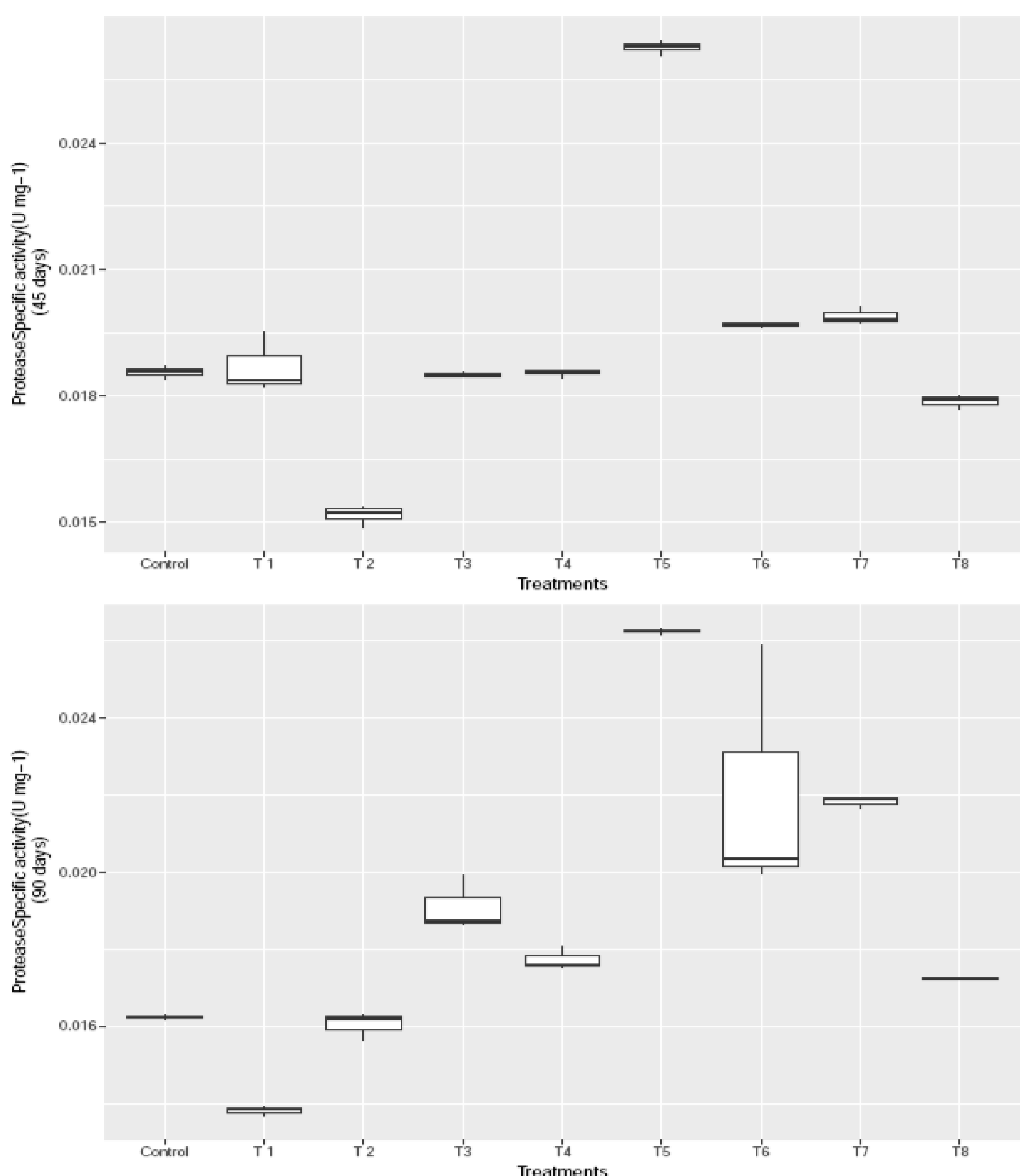


Figure 4. 22: Protease Specific activity of *L. rohita* intestinal contents after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

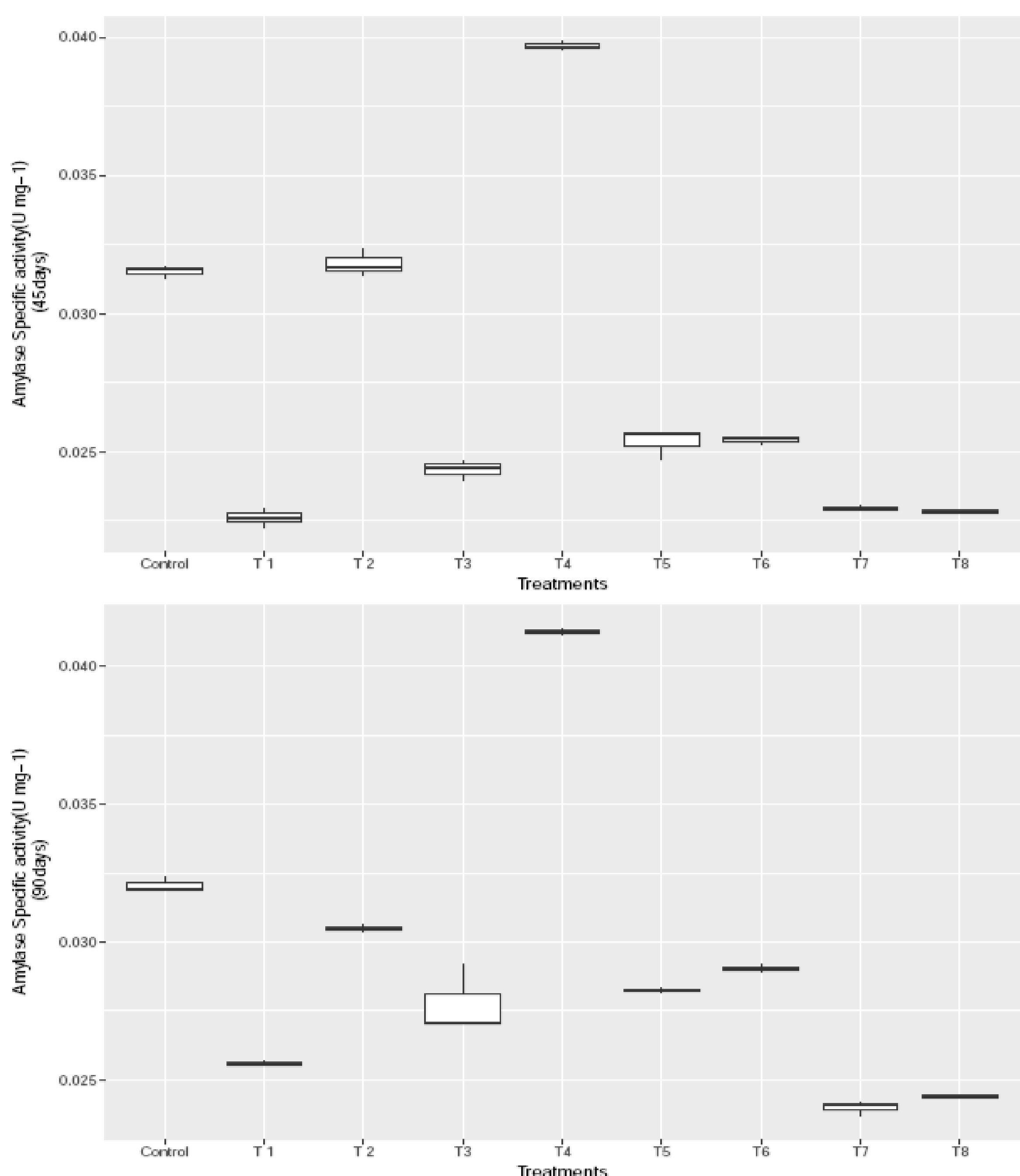


Figure 4. 23: Amylase Specific activity of *L. rohita* intestinal contents after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

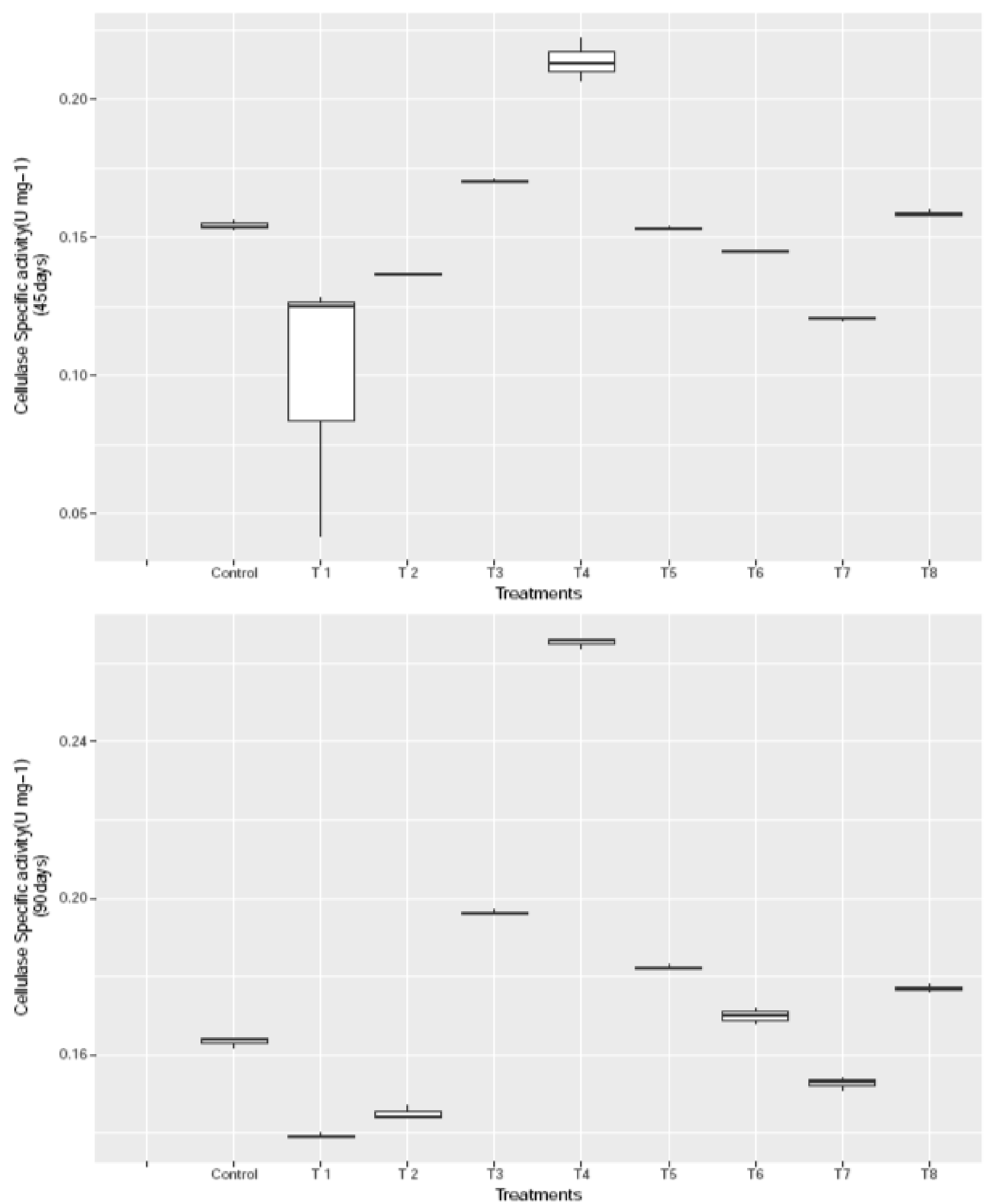


Figure 4. 24: Cellulase Specific activity of *L. rohita* intestinal contents after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data generated in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 coculture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 coculture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 coculture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

Table 4. 10: Effect of dietary administration of probiotic microorganisms (10^9 CFU gm^{-1}diet) in single and combined form on the production of digestive enzymes of *L. rohita* fingerlings.

Groups	Protease (Specific activity U mg^{-1})		Amylase (Specific activity U mg^{-1})		Cellulase (Specific activity U mg^{-1})	
	45th day	90th day	45th day	90th day	45th day	90th day
T0	0.0186 ^{cd} (±0.00008)	0.0162 ^{cd} (±0.000026)	0.025 ^{b} (±0.0001)	0.0320 ^{b} (±0.0001)	0.154 ^{bc} (±0.0009)	0.163 ^{f}(±0.0005)
T1	0.0187 ^{c} (±0.0004)	0.0138 ^{d} (±0.00006)	0.023 ^{e} (±0.0002)	0.0256 ^{f} ±(0.00004)	0.128 ^{d} (±0.002)	0.139 ^{i} (±0.000)
T2	0.0152 ^{e} (±0.0002)	0.0160 ^{cd} (±0.0002)	0.032 ^{b} (±0.0003)	0.0305 ^{e} (±0.00008)	0.137 ^{bcd} (±0.001)	0.145 ^{h} (±0.001)
T3	0.0185 ^{cd} (±0.0002)	0.0191 ^{bc} (±0.0004)	0.024 ^{d} (±0.0002)	0.0278 ^{e} (±0.0007)	0.170 ^{ab} (±0.001)	0.196 ^{b} (±0.000)
T4	0.0185 ^{cd} (±0.00005)	0.0177 ^{c} (±0.00017)	0.04 ^{a} ±(0.00001)	0.0413 ^{a} (±0.000054)	0.214 ^{a}(±0.004)	0.265 ^{a} (±0.001)
T5	0.0263 ^{a} (±0.0009)	0.0262 ^{a} (±0.00003)	0.025 ^{c} (±0.0003)	0.0283 ^{de} (±0.00003)	0.153 ^{bc} (±0.001)	0.182 ^{c} (±0.000)
T6	0.0197 ^{b} (±0.00002)	0.0221 ^{b} (±0.0019)	0.023 ^{c} (±0.00004)	0.0290 ^{d} (±0.000071)	0.145 ^{bcd} (±0.001)	0.170 ^{e} (±0.001)
T7	0.0199 ^{b} (±0.0001)	0.0218 ^{b} (±0.00009)	0.023 ^{e} (±0.00004)	0.0240 ^{g} (±0.0001)	0.121 ^{cd} (±0.001)	0.153 ^{gd}(±0.001)
T8	0.0179 ^{d} (±0.0001)	0.0172 ^{c} (±0.00001)	0.032 ^{e} (±0.00001)	0.0244 ^{fg} (±0.00)	0.159 ^{bc} (±0.001)	0.177 ^{d} (±0.00)

* **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5) *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis.**

4.9 Effect of probiotic feeding on fish nutritional profile

Nutritional composition of the *L. rohita* fingerlings after 45th and 90th days of rearing on basal and probiotic supplemented diets are shown in Table 4.11.

4.9.1 Crude protein at 45th day of feeding trial

The chemical composition analysis of dry mass of flesh of *L. rohita* at 45 day reared on *G.candidum* QAUGC01 and *E.hirae* QAUEH01 co-culture (T6) supplemented diet showed significantly higher (P<0.05) crude protein content (74.38±0.17%) as compared to all other groups. Control group contained the least crude protein percentage (66.5±0.29%).

4.9.2 Crude protein analysis at 90th day of trial

While at 90 day proximate analysis showed that fishes fed with *E. hirae* QAUEH01 (T3) and *G. candidum* QAUGC01 in combination with *E. faecium* QAUEF01 (T5) showed significantly higher ($P<0.05$) crude protein content (87.5000±0.5773) and (85.75±0.577). Least amount of crude protein percentage was observed in commercial probiotic (T8) treatment (68.25±0.309) which showed statistically non-significant variation with respect to control (T0), *G. candidum* QAUGC01(T1) and *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture (T6) while all other treatments vary significantly with respect to each other.

4.9.3 Crude fat analysis at 45th day of trial

Fats percentage at 45 day followed the same pattern with group of fish reared on diet supplemented by *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture (T6) having highest fats content of (14.5±0.06%), significantly higher than all other potential probiotic reared groups and control (9.0±0.17 %).

4.9.4 Crude fat analysis at 90th day of trial

Fat percentage content at 90 days showed that the commercial probiotic (T8) expressing maximum value (33.6±0.60) which was found to be significantly high ($P<0.05$) with respect to control and all other treatments.

4.9.5 Ash analysis (45th and 90th day)

Total ash content of a control group raised on basal diet was significantly higher ($P<0.05$) i.e., (14.0±0.24 %) as compared to all the groups of fish fed probiotic supplemented diets both at 45, same trend was followed at 90 day showing maximum ash content by control (T0) (16±0.80) but the variation among the treatments was non-significant except *E. faecium* QAUEF01 (T2) having value (12±0.57) (Table 4.11).

Table 4. 11: Effects of dietary administration of probiotic microorganisms (10^9 CFU gm^{-1}diet) in single and combined form on the whole body chemical composition ($100g^{-1}$ on dry matter basis) of *L. rohita* fingelings.

Treatments	Crude Protein (%)		Crude Fats (%)		Total Ash (%)	
	45th days	90th days	45th days	90th days	45th days	90th days
T0	66.5 c (±0.29)	70.00 d (±0.577)	9.0 c (±0.17)	18.3 e (±0.63)	14.0 a (±0.24)	16 a (±0.80)
T1	74.38 a (±0.05)	68.38 d (±0.2665)	12.3 ab (±0.12)	28.6 b (±0.64)	13.5 ab (±0.12)	15.5 a (±0.86x)
T2	74.38 a (±0.04)	78.75 c (±0.5773)	13 ab (±0.12)	14 f (±0.36)	10.86 b (±0.02)	12 b (±0.57)
T3	66.5 c (±0.23)	87.5 a (±0.5773)	11.6 bc (±0.17)	22.6 c (±0.57)	11.5 ab (±0.17)	14 ab (±0.57)
T4	66.5 c (±0.17)	83.13 b (±0.295)	12.3 ab (±0.12)	23.6 c (±0.83)	12.0 ab (±0.17)	13.2 ab (±0.83)
T5	69.13 bc (±0.17)	85.75 ab (±0.577)	10.6 bc (±0.23)	13.6 f (±0.63)	11.0 b (±0.12)	14.5 ab (±0.63)
T6	74.38 a (±0.17)	70.0 d (±0.888)	14.5 a (±0.06)	19.6 de (±0.36)	12.9 ab(±0.23)	16 a (±0.23)
T7	74.38 a (±0.23)	78.75 c (±0.498)	8.6 c (±0.29)	21.3 cd (±0.51)	12.0 ab (±0.35)	14 ab (±0.288)
T8	70.88 ab (±0.07)	68.25 d (±0.309)	8.0 c (±0.06)	33.6 a (±0.60)	12.5 ab (±0.23)	15 ab (±0.41)

* **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G .candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *E. hirae* QAUEH01 (T3), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (T6), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis.**

4.10 Imapct on Blood Glucose (45th and 90th day)

Blood glucose (mg dL^{-1}) level at 45day in all treated groups of *L. rohita* was significantly lower (P<0.05) than a group of fish fed basal diet (C, 58.2±0.12 mg dL^{-1}), the lowest value was observed in T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) group (40.37±0.07 mg dL^{-1}). Similar trend was shown at 90th day but the lowest value was observed in T6 group fed on diet supplemented with *G. candidum* QAUGC01 and *E. hirae* QAUEH01 coculture (45.23±1.12 mg dL^{-1}) as shown in (Figure 4.25). Blood glucose (mg dL^{-1}) level in all treated groups of *L. rohita* was significantly lower (P<0.05) than a group of fish fed on basal diet (C, 58.2±0.12 mg dL^{-1}), the lowest value was observed in T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) group (40.37±0.07 mg dL^{-1}).

Similar trend was observed at 90th day of experiment, lowest value was recorded by T6 (*G.candidum* QAUGC01 and *E.hirae* QAUEH01 co-culture) (45.23±1.12) and T7 (*G.candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) (45.96±0.14).

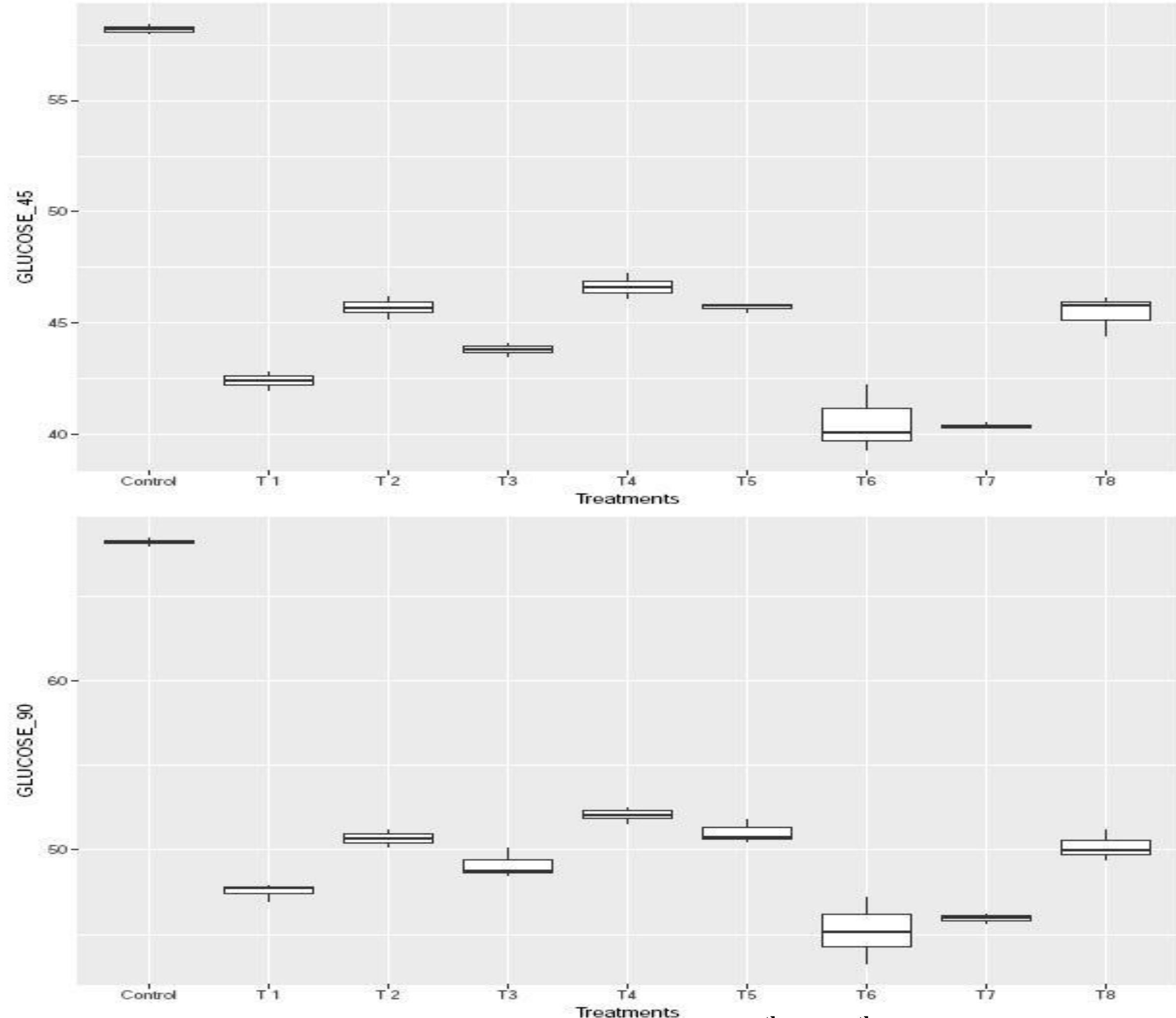


Figure 4. 25: Glucose(mg/dl) concentration of *L. rohita* after feeding for 45th and 90th day on control (basal feed) and probiotics added treatments feed (T1-T8). This is the box plot graphical respresentation of data in statistical R software.

***T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae*, QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (Commercial probiotic).This graphical data is represented as Mean ± SE (n=9).**

4.11 Challenge test with *Staphylococcus aureus* (ATCC 2593)

The fishes in control group started dying from the second day of a week long trial and all fishes were dead by the fifth day whereas the fishes fed on T7 (*G. candidum* QAUGC01

and *B. cereus* QAUBC02) were alived till the 7th day when challenged with *S. aureus* (ATCC 2593).

4.12 Correlation among physiological variables of the study (45th and 90th day)

Pearson correlation at the significance level alpha 0.05($P < 0.05$) was applied to study the correlation among different physiological parameters are shown in Table (4.12a and 4.12b)

Table 4. 12a & b: (45th day) and 4.12 b (90th day) Pearson Correlation among different physiological parameters undertaken in study at significance level P<0.05. Bold figures represent significant correlation.

WBCs= White blood cells; RBCs= Red blood cells;HGB= Hemoglobin;HCT= Hematocrit count;MCV= Mean corpuscular volume; MCH= Mean corpuscular hemoglobin; MCHC= Mean corpuscular hemoglobin concentration;PLT= Platelets;LYM= Lymphocytes;SGR= Specific growth rate; FCR= Feed conversion ratio; FCE= Feed conversion efficiency.

	Variables	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	WBCs $\times 10^3/\mu l$	-	**.47**	**.75**	.33	0.00	0.25	0.06	**0.65**	0.01	**0.54**	**0.51**	-0.04	0.07	**-0.41**	-0.09
2	RBCs $\times 10^6/\mu l$			**.55**	**.75**	0.23	**-.54**	**-.47**	-0.14	.23	.25	.25	-0.23	0.37	-0.14	0.21
3	HGB g/dl				.31	-.16	0.15	.12	.32	-.22	**.57**	**.55**	-0.32	**0.39**	-0.28	-.15
4	HCT%					**.77**	**-.69**	**-.83**	-.32	.28	-.21	-0.22	0.18	-.11	-.14	0.66
5	MCVfl						**-.56**	**-.87**	**-.40**	0.25	**-.67**	**-.68**	**0.54**	**-.48**	-0.07	**.72**
6	MCH pg							**.86**	**0.52**	**-0.39**	.32	.30	-0.19	.15	-.25	**-.53**
7	MCHC g/dl								**.45**	-.36	**.53**	**.53**	**-.39**	.34	-0.09	**-0.61**
8	PLT×103/µl									0.07	**0.56**	**0.56**	.10	-0.13	-.21	**-.47**
9	LYM%										0.03	0.05	.18	-.23	0.29	0.19
10	%Growth											**.99**	**-.39**	**.38**	0.15	**-.47**
11	SGR												**-.39**	**0..39**	.15	**-.47**
12	FCR													**-.98**	0.00	**.48**
13	FCE														-0.04	**-.42**
14	Protease															-.26
15	Amylase															-

	Variables	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	WBCs × 10^3/µl	-	**.91**	**.88**	**.55**	**-.39**	**-.53**	**.40**	**.55**	**.94**	**.44**	**.43**	.11	-.09	-.11	-.14
2	RBCs × 10^6/µl			**.96**	**.71**	-.20	**-.44**	.32	.35	**.80**	**.55**	**.48**	.09	-.07	.16	-.14
3	HGB g/dl				**.73**	-.17	-.35	.31	.33	**.75**	**.57**	**.51**	.07	-.06	.12	-.20
4	HCT%					**.50**	-.31	-.37	-.29	**.45**	**.46**	.30	-.11	.13	.15	-.05
5	MCVfl						.11	**-.94**	**-.91**	**-.39**	-.03	-.13	-.25	.26	.14	.16
6	MCH pg							-.03	-.18	**-.65**	.15	.09	-.36	.34	.26	-.37
7	MCHC g/dl								**.83**	.33	.19	.19	.25	-.26	.08	-.25
8	PLT×103/µl									**.55**	.15	.23	.16	-.17	-.26	-.33
9	LYM%										.23	.28	.16	-.14	-.32	.00
10	%Growth											**.69**	**-.47**	**.46**	**.40**	**-.73**
11	SGR												-.34	.34	.27	**-.56**
12	FCR													**.99**	.16	**.44**
13	FCE														**-.17**	**-.41**
14	Protease															-.12
15	Amylase															-

Phase –III Impact Feeding Probiotics on Gut Microbial Modulation

4.12 Gut modulation by probiotics (Culture Dependent Method Vs Advance Molecular Techniques)

4.12.1 Impact of probiotic feeding on gut microbiology by culture dependent method

The impact of probiotic feeding on the fish gut microbiology was monitored by culturing on Tryptic soy agar (TSA), M17medium, De Man, Rogosa and Sharpe agar (MRS) , MacConkey agar and Oxytetracycline agar medium(OGA).

After 24-48 hours incubation media plates were observed to check the growth of microorganisms (Figure 4.26a, 4.26b, 4.26c and 4.26d). The colonies that appeared on TSA plates were minute, small as well as large size circular and irregular colonies of white and pale color having two types of margins entire and undulate. Colonies produced on M17 agar plates showed minute, small and large circular colonies were present with pale yellow and white color and even margins. MRS plates produced pin point and small off white, white and pale colonies. MacConkey plates showed lactose positive (Yellow) as well as lactose negative (Pink) colonies of various sizes (minute, small and big). Some colonies on MacConkey plates were having rough margins while some were having smooth margins. Small whitish colonies of uniform sizes and some samples showed fluffy white wooly colonies were gorwn on OGA plates (Table 4.13).

Table 4. 13: Macroscopic characterization of colonies on growth media after 24-48hrs of incubation at 37°C

Microbial type/Media type	**Colony shape**	**Colony size**	**Colony color**	**Colony margin**	**Colony surface**
Total bacterial counts (TSA)	Circular Irregular	Minute, small and large	Milky white pale	Entire Undulate	Raised flat
Enterococcus / Streptococcus (M17)	CircularCircular	Pinpointed, small ,large	Pale yellow white	Entire/ Smooth	Flat
Lactic acid bacteria (MRS)	Circular	Pinpoint small	Off white pale	Entire/ Smooth	Flat
Lactic acid bacteria (MRSA)	Circular	Minute, small , large	Yellow (Lac+) Pink (Lac-)	Entire Undulate	Flat raised
Fungi (OGA)	Circular	Small	White	Entire	Fluffy convex

**Lac+(Lactose +ve) and* Lac-(Lactose-ve)

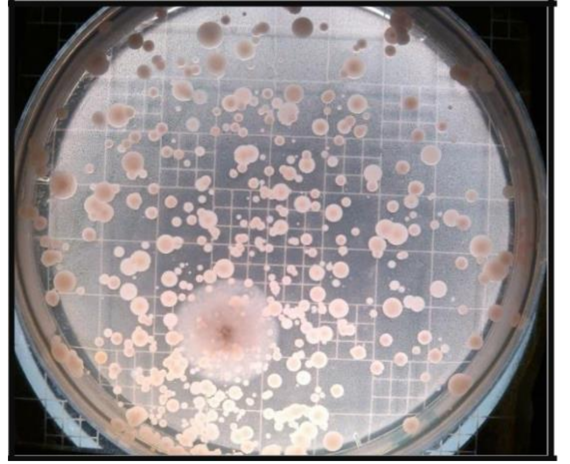


Figure 4.26 (a) A photograph showingTSA medium culture plate

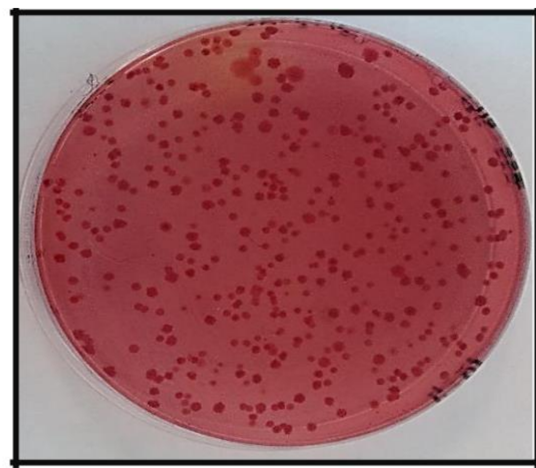


Figure 4.26 (b) A photograph showing.Macconkey medium culture plate

Figure 4.26(c) A photograph showing OGA medium culture plate

Figure 4.26 (d) A photograph showing M17 medium culture plate

Figure 4. 26: Microbial growth of fish gut microbes on specific media 4.12.2 Microscopic Examination

Microbial colonies on different media were microscopically examined by using Gram's staining and lacto phenol cotton blue staining. There were Gram positive cocci, Gram negative Bacilli as well as Gram positive Bacilli on TSA. M17 colonies were Gram positive cocci, MRS colonies were Gram positive Bacilli. On MacConkey Agar there were Gram

negative Bacilli. Yeast colonies from OGA media were examined by Lactophenol cotton blue (Figure 4.27a, 4.27b, 4.27c and 4.27d).

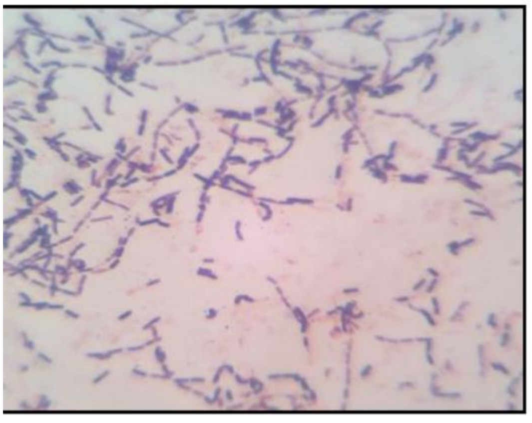


Figure 4.27 (a) Gram positive Bacilli

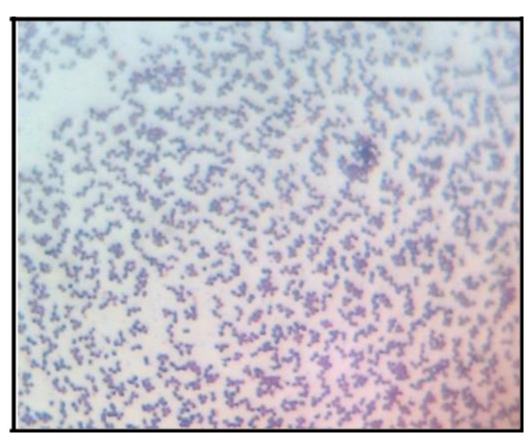


Figure 4.27 (b) Gram positive Cocci

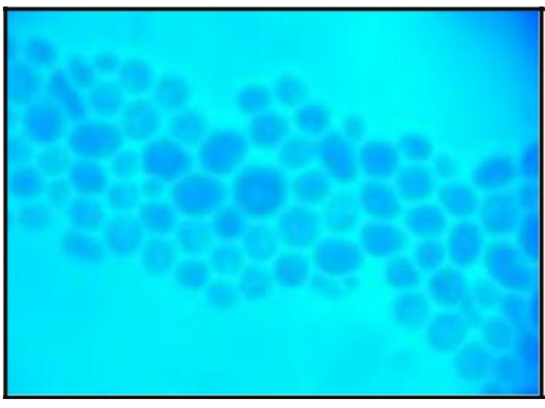


Figure 4.27(c) Yeast

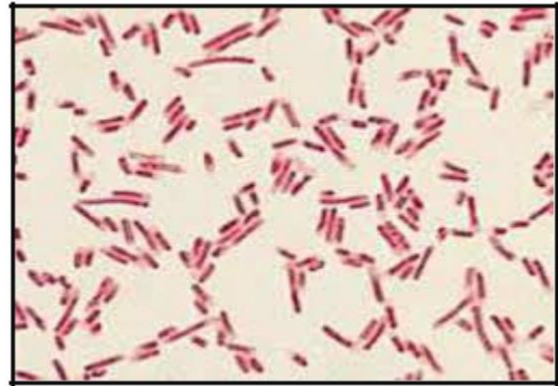


Figure 4.27(d) Gram negative Bacillus

Figure 4. 27: Stianing results of bacteria and yeast

4.13 Quantification of Microbiota in Fish Gut (cfu/gm of intestinal contents)

Enumeration of *L. rohita* intestinal gut microbiome was done on five different media, This culture dependent method was used for estimation of gut microbiology.

4.13.1 Enumeration of total arobic bacteria (TABC)

TSA is a general purpose media used for overall count of microorganisms in intestinal sample. Total count on TSA media was ranged from 1.92×10^6 – 1.01×10^8. Gut microbiome total count of *L. rohita* was found 5.93×10^7, 3.43×10^7, 1.47×10^7, 1.71×10^7, 7.23×10^7, 1.92×10^6, 2.59×10^6, 1.01×10^8 and 1.33×10^7 cells /gm in T0 – T8 group respectively after 90 days trial. Highest number of bacterial count was observed in T7

which was 1.01×10^8 while lowest value observed in T5 was 1.92×10^5. This data is graphically represented in Figure 4.28.

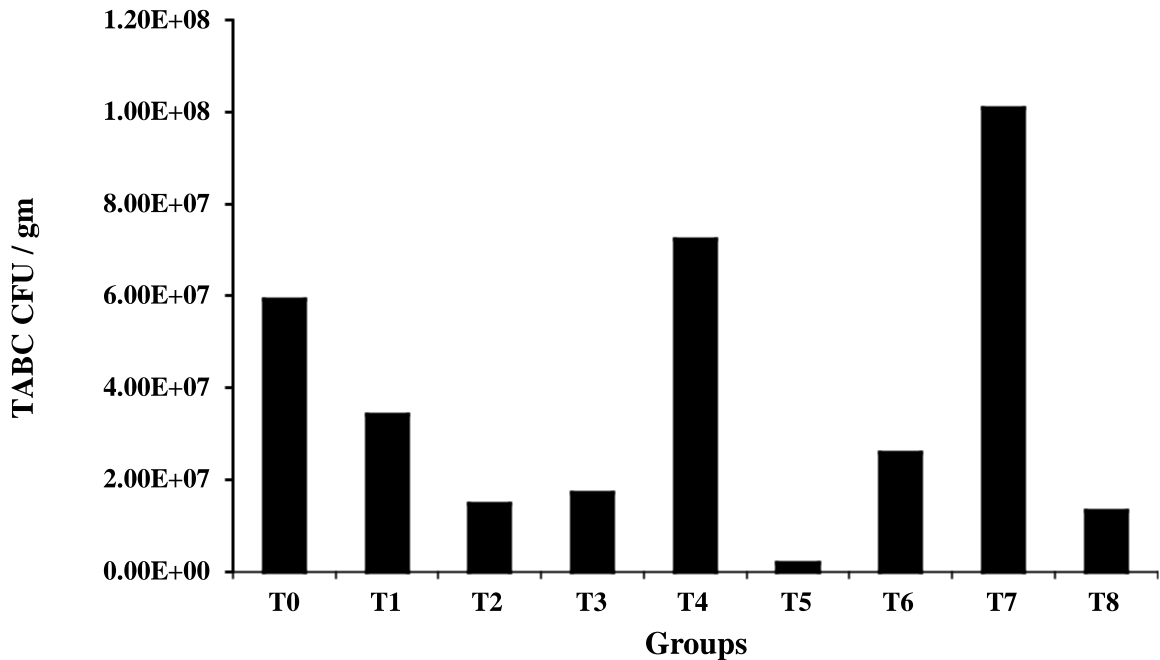


Figure 4. 28: Impact of probiotics feeding on total aerobic bacterial count (TABC) of *L. rohita* gut. This data is represented in CFU/gm form* TABC(Total aerobic bacterial count).

4.13.2 Enumeration of Lactococcus and Enterococcus count

This media was used to check the impact of feeding probiotics on *Lactococcus*, *Streptococcus* and *Enterococcus* diversity of *L. rohita* gut by culturing method. Assumed count of *Lactococcus*, *Streptococcus* and *Enterococcus* was 9.59×10^7 (T0), 5.82×10^7 (T1), 2.71×10^7 (T2), 5.00×10^5 (T3), 6.09×10^7 (T4), 1.55×10^7 (T5), 1.73×10^7 (T6), 2.87×10^8 (T7) and 1.42×10^7 (T8) respectively. Assumed count range was recorded between $5.00 \times 10^5 – 2.87 \times 10^8$. Highest count was recorded in T7 group fed with (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) and lowest in T3 fed with *E. hirae* QAUEH01 (Figure 4.29).

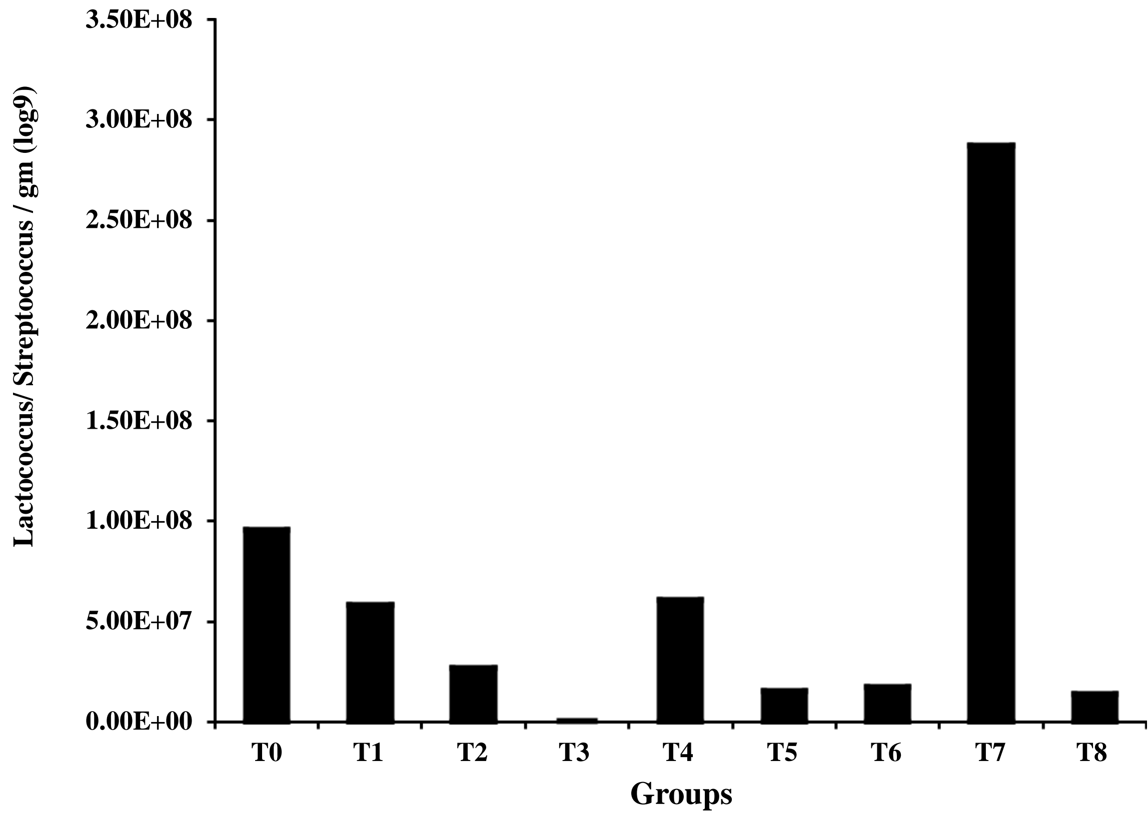


Figure 4. 29: Impact of feeding probiotics on Lactococcus, Streptococcus and Enterococcus count of L. rohita gut. This data is represented in CFU/gm (log ^{9}) form.

14.3.3 Enumeration of Lactobacillus

MRS media was used to check the impact of feeding probiotics on lactic acid bacterial diversity of *L. rohita* gut. Assumed lactobacillus count of treated and control groups T0-T8 was 4.64×10^6, 9.92×10^7, 5.96×10^6, 1.52×10^6, 4.45×10^6, 8.92×10^6, 3.91×10^6, 1.68×10^6 and 4.45×10^6 respectively. Highest count was 9.92×10^7 T1 group fed with (*G. candidum* QAUGC01), while lowest count was in T3 group fed with (*E. hirae* QAUEH01). Graphical representation of data is in (Figure 4.30).

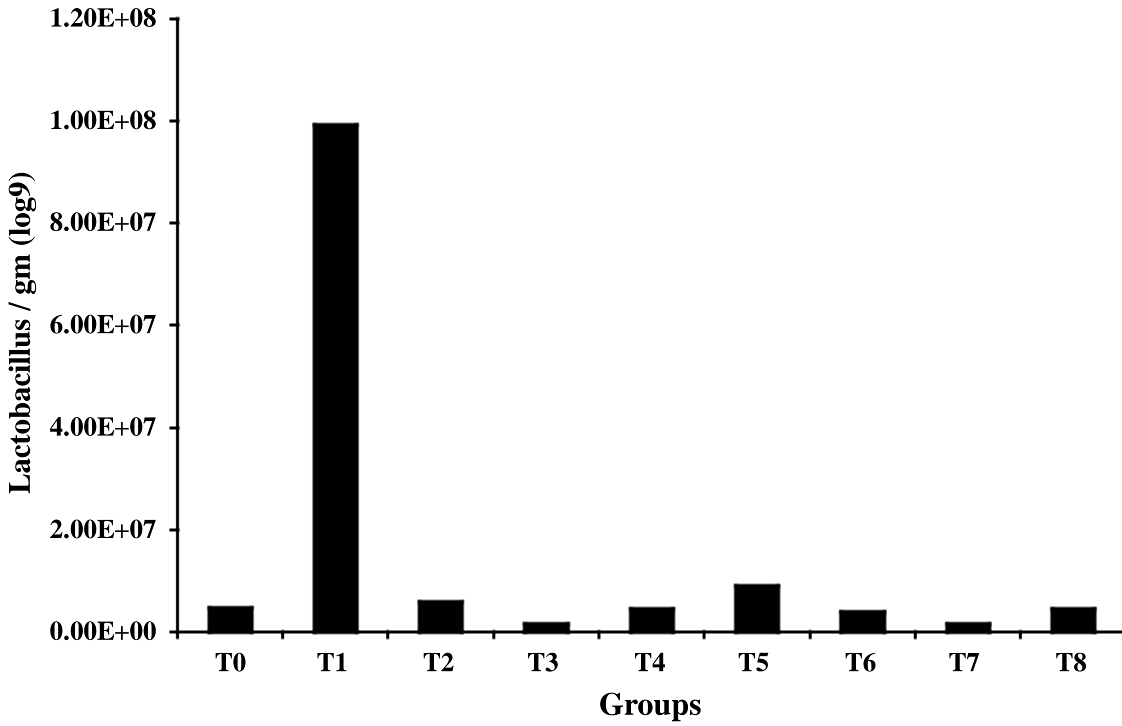


Figure 4. 30: Impact of feeding probiotics on Lactobacillus diversity count of *L. rohita* gut. This data is represented in CFU/gm (10^9).

4.13.4 Enumeration of Enterobacteriaceae

In despondence of 90 days probiotics feeding trial MacConkey agar was used to check the Coliforms count of *L. rohita* gut. Highest Coliforms count 1.64×10^7 was observed in T0 control group fed with basal diet, while lowest count was 1.18×10^6 in T2. All groups fed with probiotics supplemented diet showed significantly lower number of Coliforms as compared to control fed with basal diet. Graphical representation of data is in (Figure 4.31)

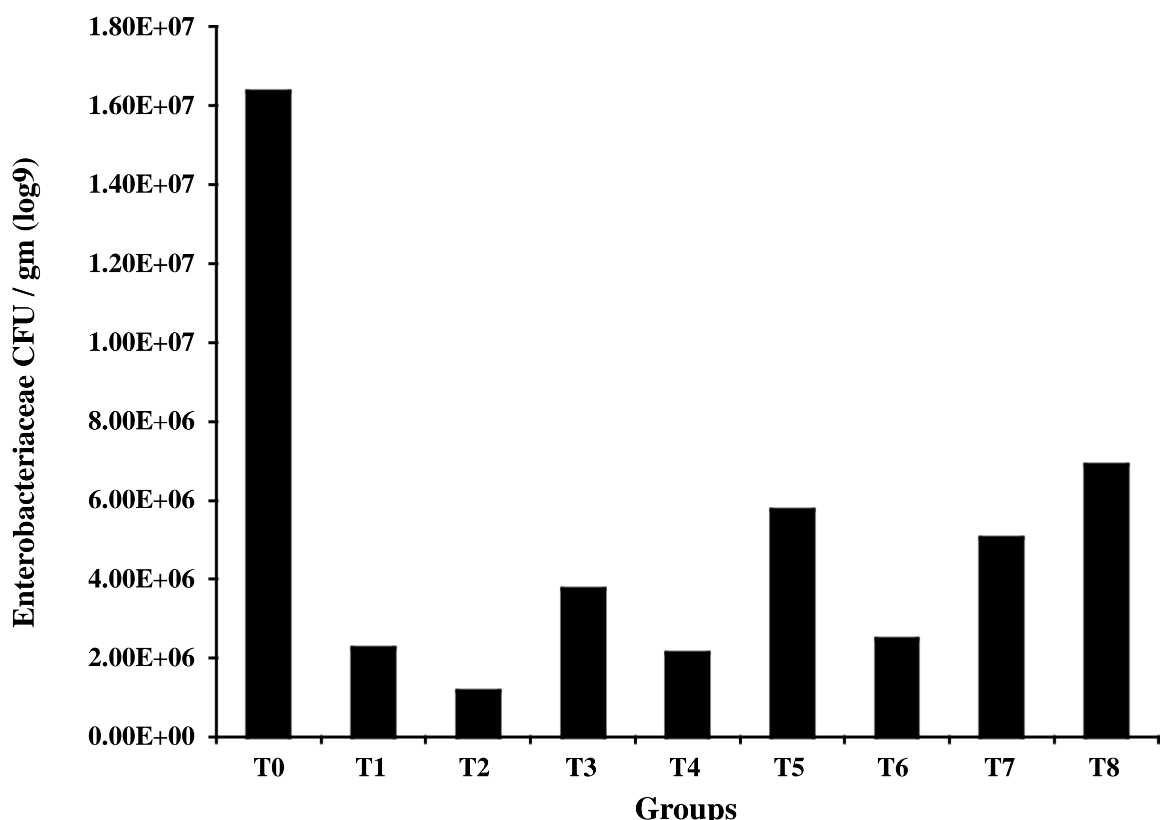


Figure 4. 31: Impact of feeding probiotics on coliforms diversity count of *L. rohita* gut. This data is represented in CFU/gm (log9) form.

4.13.5 Total yeast count

OGA media was used to evaluate the yeast diversity of *L. rohita* gut after probiotics supplemented feed application trial. Yeast count of the groups was found between $3.33 \text{ x}10^5$ – 8.41×10^7. Assumed yeast count in experimental groups was 3.64×10^5 (T0), 8.41

$\times 10^7$ (T1), 2.26 $\times 10^6$ (T2), 5.83 $\times 10^5$ (T3), 6.91 $\times 10^7$ (T4), 3.33 $\times 10^5$ (T5), 1.91 $\times 10^6$ (T6), 3.77 $\times 10^6$ (T7) and 4.29 $\times 10^5$ (T5). Highest yeast count was observed in T1 group fed with (*G. candidum* QAUGC01) and lowest was in T5 fed with (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture) (Figure 4.32)

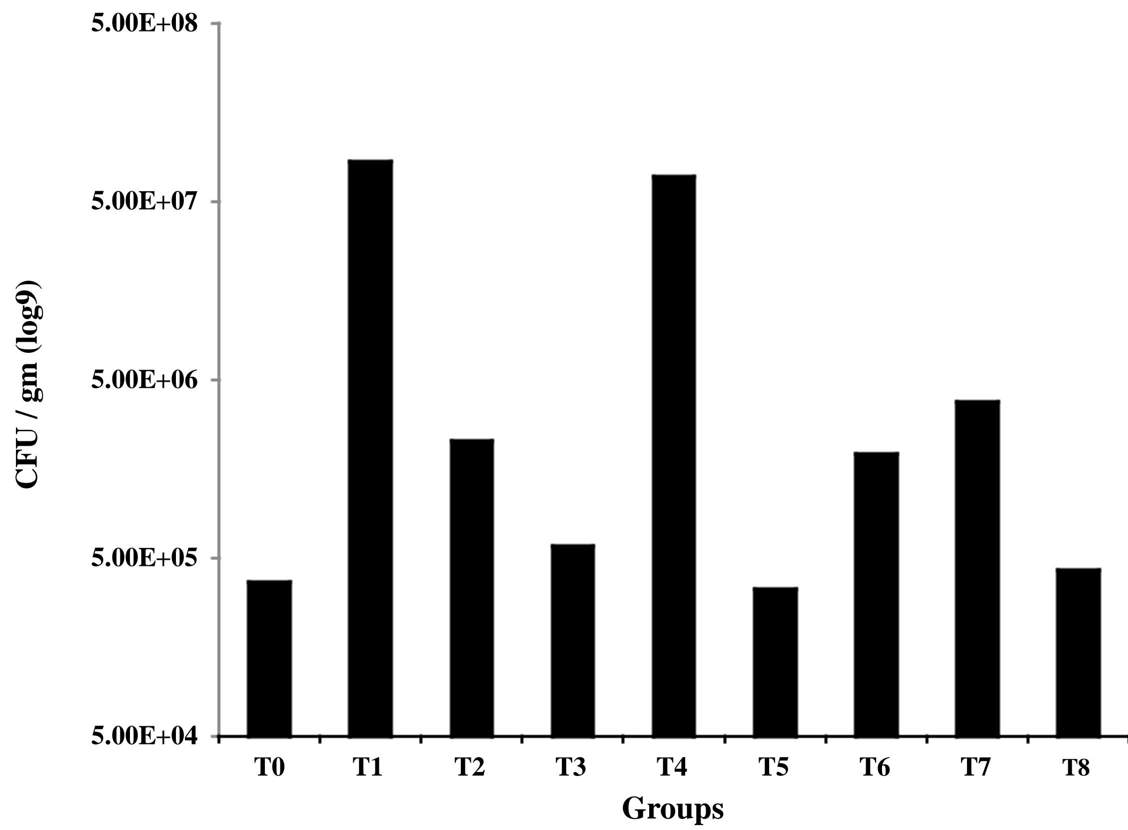


Figure 4. 32: Impact of feeding probiotics on Yeast diversity count of *L. rohita* gut. This data is represented in CFU/gm (log^9) form.

Table 4. 14: Cumulative table showing cfu/gm values of treatments based on culturing techniques

Sr. no.	Treatments	Culturing media				
		TSA	MC	M17	MRS	OGA
1	T0 (control)	5.93E+07	1.64E+07	2.87E+08	4.64E+06	3.64E+05
2	T1	3.43E+07	2.25E+06	5.82E+07	9.92E+07	8.41E+07
3	T2	1.49E+07	1.18E+06	2.71E+07	5.96E+06	2.26E+06
4	T3	1.71E+07	3.75E+06	5.00E+05	1.52E+06	5.83E+05
5	T4	7.23E+07	2.14E+06	6.09E+07	4.45E+06	6.91E+07
6	T5	1.92E+06	5.75E+06	1.55E+07	8.92E+06	3.33E+05
7	T6	2.59E+07	2.48E+06	1.73E+07	3.91E+06	1.91E+06
8	T7	1.01E+08	5.05E+06	9.59E+07	1.68E+06	3.77E+06
9	T8	1.33E+07	6.91E+06	1.42E+07	4.45E+06	4.29E+05

*TSA (Tryptic soy agar), MC (MacConkey agar), M17 (M17 agar), OGA (Oxytetracycline agar medium), T0 (Basal diet), T1(*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae* QAUEH01), T4 (*B. cereus* QAUBC02), T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01), T6 (*G. candidum* QAUGC01 co-culture with *E. hirae* QAUEH01), T7 (*G. candidum* QAUGC01co-culture with *B. cereus* QAUBC02),T8(commercialprobiotic).

4.14 Evaluation of the Impact of probiotics feeding on gut microbial diversity by culture independent method

4.14.1 Microbial diversity by 16S rDNA based metagenomics

DNA from intestinal samples was extracted by using *Favor Prep Stool DNA Isolation Mini Kit* (FAVORGEN) as per their recommended protocol. The seven treatments that were selected for metagenomics analysis were the ones that showed more promising impacts on the studied physiological parameters.

Qualitative confirmation of extracted DNA was done by using gel electrophoresis. Quantification and purity of extracted DNA assessed by Nano drop is given in the Table 4.15. DNA quantity in all samples was sufficient to the requirements for the further processing of samples for metagenomics analysis. Highest DNA quantity was in T0

(445ng). T1(*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02)samples DNA was pure as while in T0 and T8 there was a little impurity of salts or chemical compounds (Table 4.15).

Table 4. 15: Gut microbial flora extracted DNA quantity and purity level after 90th day of probiotics feeding in *L. rohita*

Samples	ng/µl	A260	A280	260/280
T0	445	8.912	7.077	1.26
T1	107.24	2.145	1.138	1.88
T2	12.65	0.131	0.064	2.03
T4	14.23	3.345	2.061	1.62
T5	109.84	2.197	1.140	1.93
T7	187.63	3.753	2.071	1.81
T8	9.48	0.190	0.123	1.54

* ng/µl (nano gram /micro litre), A260 (Absorbance at 260nm), A280 (Absorbance at 280nm).

** **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *B. cereus* QAUBC02 (T4), *G.candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis.**

4.15 Gut microbial diversity by 16S rDNA based Metagenomics analysis

4.15.1 Differential analysis at Phylum level

The control group (T0) showed six phyla, where Proteobacteria (97.74%), Actinobacteria (1.28%), Firmicutes (0.46%), Planctomycetes (0.39%), Bacteroidetes (0.07%) and Chlamydia (0.001%). The T4 group showed maximum 9 phyla, where Proteobacteria (97.98%), Actinobacteria (1.39%), Firmicutes (0.20%), Planctomycetes (0.25%), Bacteroidetes (0.05%), Chlamydia (0.04%), Verrucomicrobia (0.05%), Spirochaetes (0.001%) and Tenericutes (0.001%). T1 group represented 4 phyla, where Proteobacteria (99.70%), Firmicutes (0.25%), Actinobacteria (0.03%) and Bacteroidetes (0.005%). T2 fed on diet supplemented with (*G.candidum* QAUGC01 and *E.faecium* QAUEF01 coculture)

showed 7 phyla that included Proteobacteria (0.87%), Actinobacteria (0.05%), Firmicutes (99.04%), Planctomycetes (0.0032%), Bacteroidetes (0.008%), Cyanobacteria (0.0048%), Spirochaetes (0.0032%), T5 contains six phyla where Proteobacteria (99.69%), Actinobacteria (0.047%), Firmicutes (0.24%), Planctomycetes (0.0075%), Bacteroidetes (0.0075%) and Cyanobacteria (0.0015%). Group T7 fed with mixed probiotics represent 5 phyla where, Proteobacteria (99.66%), Firmicutes (0.27%), Actinobacteria (0.04%) and Bacteroidetes (0.008%) and Spirochaetes (0.004%). T8 (commercial probiotic represented 6 phyla that included Proteobacteria (84.57%), Actinobacteria (11.03%), Firmicutes (0.263%), Bacteroidetes (0.10%), Cyanobacteria (4.015%), Spirochaetes (0.0035%).

Control group showed 5 phyla where Ascomycota (98.50%), Basidiomycota (1.48%), Neocallimastigomycota (0.002%), Glomeromycota (0.001%) and Cryptomycota (0.0005%). T4 (*B.cereus* QAUBC02) group represented 5 phyla where Ascomycota (99.28%), Basidiomycota (0.68%), Neocallimastigomycota (0.002%), Glomeromycota (0.01%) and Cryptomycota (0.01%).T1 group represented 4 phyla Ascomycota (99.46%), Basidiomycota (0.49%), Neocallimastigomycota (0.02%), Glomeromycota (0.007%). T2 represent 3 phyla that included Basidiomycota (97.59%), Ascomycota (2.39%), Neocallimastigomycota (0.0019%). T5 treatment showed two phyla Basdiomycota (0.59%) and Ascomycota (99.40%). T8 represent 4 phyla Crytomycota (0.0051%), Basidiomycota (28.70%), Ascomycota (71.28%) and Neocallimastigomycota (0.0060%) T7 group showed just 2 phyla Ascomycota (98.40%) and Basidiomycota (0.59%). In both probiotics treated and control group fed with basal diet, Ascomycota phylum class Sacharomycetes was dominated.

4.15.2 Comparative diversity analysis at specie level

There were total 107 species in T4 while 94 species in T0. After omitting species with relative abundance (< 0.1) 14 species remained in T4 and 19 in T0. Dominated species were *Pseudomonas psychrophila* in T0 (87.21%), T4 (86.53%), *Pseudomonas plecoglossicida* T0 (0.82%), T4 (0.40%), *Pseudomonas* sp T0 (0.51%), T4 (0.48%), *Achromobacter xylosoxidans* T0 (0.32%), T4 (0.28%), *Pseudomonas trivialis* T0 (0.44%), T4 (0.46%) *Pseudomonas syringae* T0 (2.18%), T4 (3.12%), *Pseudomonas fragi* T0 (3.87%), T4 (5.64%), *Acidothermus cellulolyticus* T0 (0.95%), T4 (1.22%), *Rhodobacter* sp T0 (0.52%), T4 (0.29%), *Mycobacterium sp* T0 (0.12%), *Clostridium* sp. T0 (0.14%),

Rhodopseudomonas palustris T0 (0.21%), *Singulisphaera* sp T0 (0.16%), *Bosea thiooxidans* T0 (0.27%) and *Staphylococcus saprophyticus* T0 (0.15%). The genus *Pseudomonas* dominantly recovered from both T0 and T4. There were 80 species in T2 after omitting with relative abundance (<0.1) 7 species remained in T2. Dominated species were *Paenibacillus lactis* (85.83%), *Bacillus szutsauensis* (12.74%), *Achromobacter xylosoxidans* (0.19%), *Pseudomonas psychrophila* (0.163%), *Paenibacillus sp.* (0.123%), and *Pseudomonas trivialis* (0.106%). There were 75 species in T5 after omitting with relative abundance (<0.1) 13 species remain in T5. Dominated species were *Achromobacter xylosoxidans* (50.80%), *Klebsiella oxytoca* (25.92%), *Serratia quinivorans* (13.17%) and *Raoultella ornithinolytica* (4.35%). T8 (commercial probiotic) contained 56 species after omitting species with relative abundance (< 0.1) 24 species remained in T8 treatment. Dominated species were *Pseudomonas psychrophila* (24.04%), *Pseudomonas plecoglossicida* (12.99), *Pseudomonas* sp. (9.88%) and *Klebsiella oxytoca* (3.32%).

There were total 63 in T1 and 64 species in T7. After omitting species with relative abundance (<0.1) 13 species remained in T1 and 14 in T7. Dominated species were *Achromobacter xylosoxidans* representing T7 (48.89%), T1 (51.82%), *Klebsiella oxytoca* T7 (25.53%), T1 (25.88%), *Serratia quinivorans* T7 (12.53%), T1 (13.06%), *Raoultella ornithinolytica* T7 (4.34%), T1 (4.38%), *Pseudomonas trivialis* T7 (3.82%), T1 (0.17%), *Achromobacter* sp. T7 (2.82%), T1 (2.70%), *Stenotrophomonas* sp T7 (0.22%), T1 (0.44%), *Pseudomonas psychrophila* T7 (0.29%), T1 (0.23%), *Enterobacter* sp. T7 (0.23%), T1 (0.22%), *Bacillus szutsauensis* T7 (0.17%) and *Paenibacillus lactis* T7 (0.12%), T4 (0.10%). Dominant genus in T7 and T1 was *Achromobacter.*

Phylogenetic classification at species level at 3% divergence (97% similarity) OTUs were classified in 35 to 57 fungal species in each sample while 64 species collectively. T0 and T4 showed higher number of species as compare to T1 and T7, there were total 51 in T4 while 57 species in T0. After omitting species with relative abundance (< 0.1) 10 species remained in T4 and 11 in T0. Control group (T0) and T4 were dominated by *Debaryomyces hansenii* T0 (88.61%), T4 (97.22) followed by, *Knufia epidermidis* T0 (2.09%), T4 (0.16%), *Galactomyces candidum* T0 (0.50%), T4 (0.41%), *Galactomyces geotrichum* T0 (0.55%), T4 (0.54), *Wallemia* sp T0 (0.19%), T4 (0.16%), *Cryptococcus magnus* T0 (0.18%), T4 (0.16), *Trichoderma longibrachiatum* T0 (4.11%), *Curvularia cochliobolus*

lunatus T0 (1.95%) and *Malassezia restricta* T0 (1.00%). The genus *Debaryomyces* dominantly recovered from both T0 and T4, which depict that feeding of *B. cereus* QAUBC02 in single form did not alter the gut microbiology profile.

T1 and T7 showed reduced richness at species level same as in bacterial community as compare to T0 and T4, there were total 35 species both in T1 and T7. After omitting species with relative abundance (<0.1) 9 species remained in T1 and 10 in T7. T1 and T7 groups were dominated by *Galactomyces candidum* T7 (36.32%), T1 (38.09%), followed by *Galactomyces geotrichum* T7 (30.18%), T1 (28.85%), G*alactomyces* sp. T7 (24.32%), T1 (23.55%), *Fusarium gibberella fujikuroi* T7 (3.28%), T1 (4.155), *Debaryomyces hansenii* T7 (2.95%), T1 (2.40%), *Meyerozyma guilliermondii* T7 (1.60%), T1 (1.80%), *Wallemia* sp. T 7 (0.21%), T1 (0.14%) and *candida* sp T7 (0.21%), T1 (0.20%) (Table 4.16).

Table 4. 16: Diversity measure number of analyzed sequences, Diversity, richness (OTUs) and diversity index (Shannon and Simpson) for 16S rRNA sequencing libraries of treated and control samples.

Sample ID	No. of reads		No. of OTUs		Shannon Index		Simpson Index		Observed Species	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
T0	67926	381936	158	169	0.70	0.57	0.23	0.78	158	169
T1	71409	14125	150	95	1.33	1.44	0.64	0.71	150	95
T2	71030	52595	133	70	0.13	0.49	0.03	0.23	133	70
T4	68914	468409	177	160	0.67	0.20	0.24	0.05	177	160
T5	66219	15672	167	97	1.36	1.48	0.65	0.72	167	97
T7	46598	17143	146	96	1.44	1.44	0.67	0.71	146	96
T8	27725	65975	91	109	2.55	1.26	0.88	0.54	91	109

***OTUs(operational taxonomic units)**

** **Basal diet taken as control (T0) , single/Mix Strain probiotic supplemented feed, *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *B. cereus* QAUBC02 (T4), *G. candidum* QAUGC01 and *E. faecium* QAUEF01 (T5), *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) and commercial probiotic consortia (T8). This data is represented in the form of Mean ± SE (n=9). Different alphabet above the mean values in the columns show that they are significantly different i.e. (P<0.05) ANOVA followed by Duncan and Tukey analysis.**

The hierarchal clustering based on bacterial metagenomics as illustrated statistically by heatmap represented that the T5 and T7 showed maximum similarity in their microbial community structure as compared to other treatments. T0 and T4 also showed few

similarities in their microbial diversity. T2, T4 and T5 shares few similarities and T0 and T8 showed dissimilar microbial communities (Figure 4.33 a).

Statistical analysis of fungal metagenomics analysis showed that T5 and T7 showed first order clustering sharing maximum similarity, second order clustering is showed by T1 and third order clustering with T0 which meant very few similarities among them. T4 and T8 are also showing less similarities, T2, T4 and T8 are also showing dissimilarities (Figure 4.33 b).

The shared and unique OTUs among the different dietary groups were also represented by a Venn diagram. The bacterial genera shared between T0, T1, T2 and T4 are 22 (18.8%). The unique genera found in T0 were 9 (7.7%), 5 by T1 (4.3%), 9 (7.7%) in T2, 17 in T4 (14.5%). 20 genera (20.6%) similarity between T0, T5,T7 and T8. 29 (29.9%) unique genera were were observed by T0, 4 (4.1%) by T5, 3 (3.1%) by T7, 5 (5.2%) by T8 (Figure 4.34a and 4.34b).

The fungal genera are shared among the treatments T0, T5, T7 and T8 are 23 (53.5%). The unique found are 4 (9.3%) in T0, 1 (2.5%) in T5, 1 (2.5%) in T7 and 0 (0%) in T8. The fungal genera which are shared among treatments T0, T1, T2 and T4 are 22(50%). Unique genera showed by T0 are 3 (6.8%), T1 (0) %, T4 are 2(4.5%), T2 is 1(2.5%). (Figure 4.35a and 4.35b).

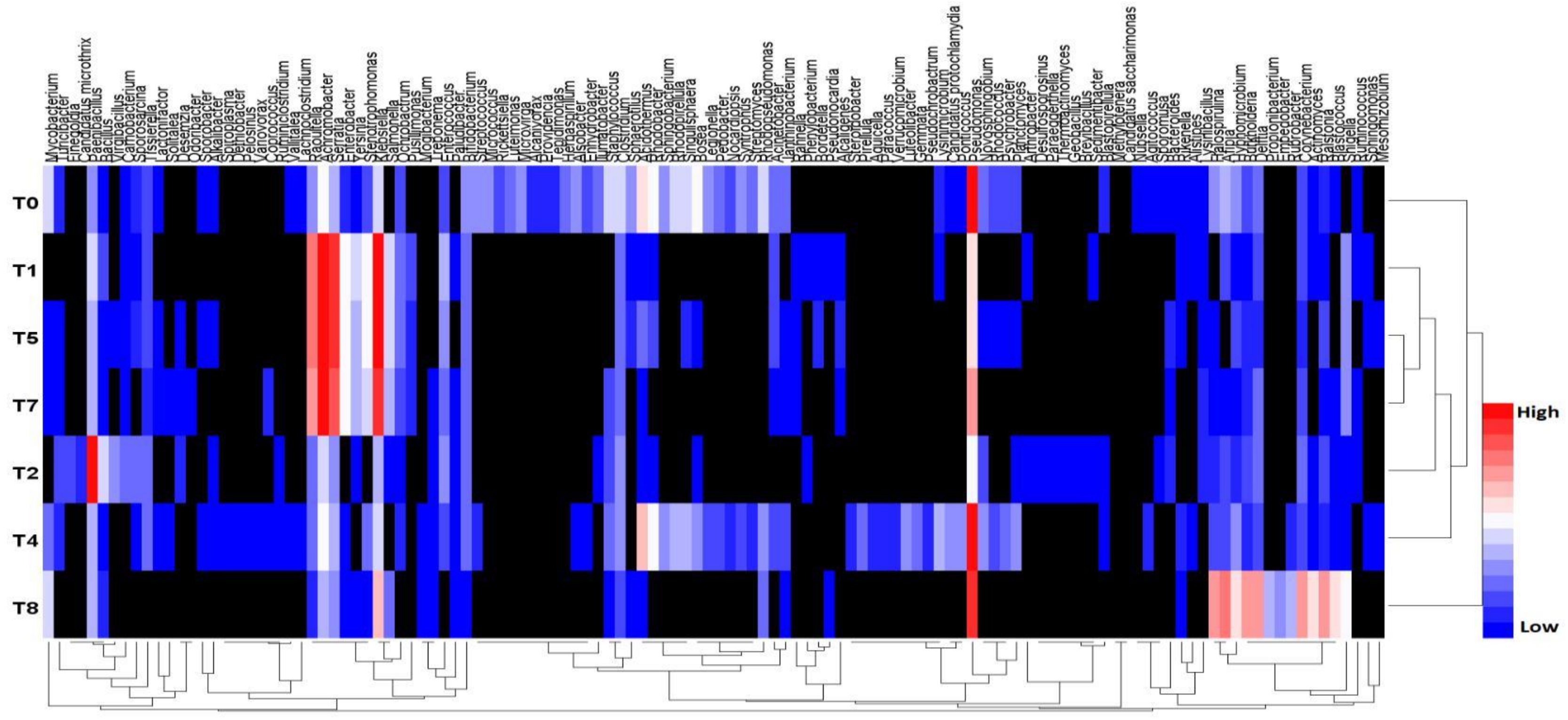


Figure 4. 33 a: Heat maps analysis of bacterial community structure generated from gut metagenomics of *L. rohita* based on clustering levels .

***T0 (Basal diet taken as control), T1 (*G. candidum* QAUGC01), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T2 (*E. faecium* QAUEF01),T4 (*B. cereus* QAUBC02), T8 (Commercial probiotic).**

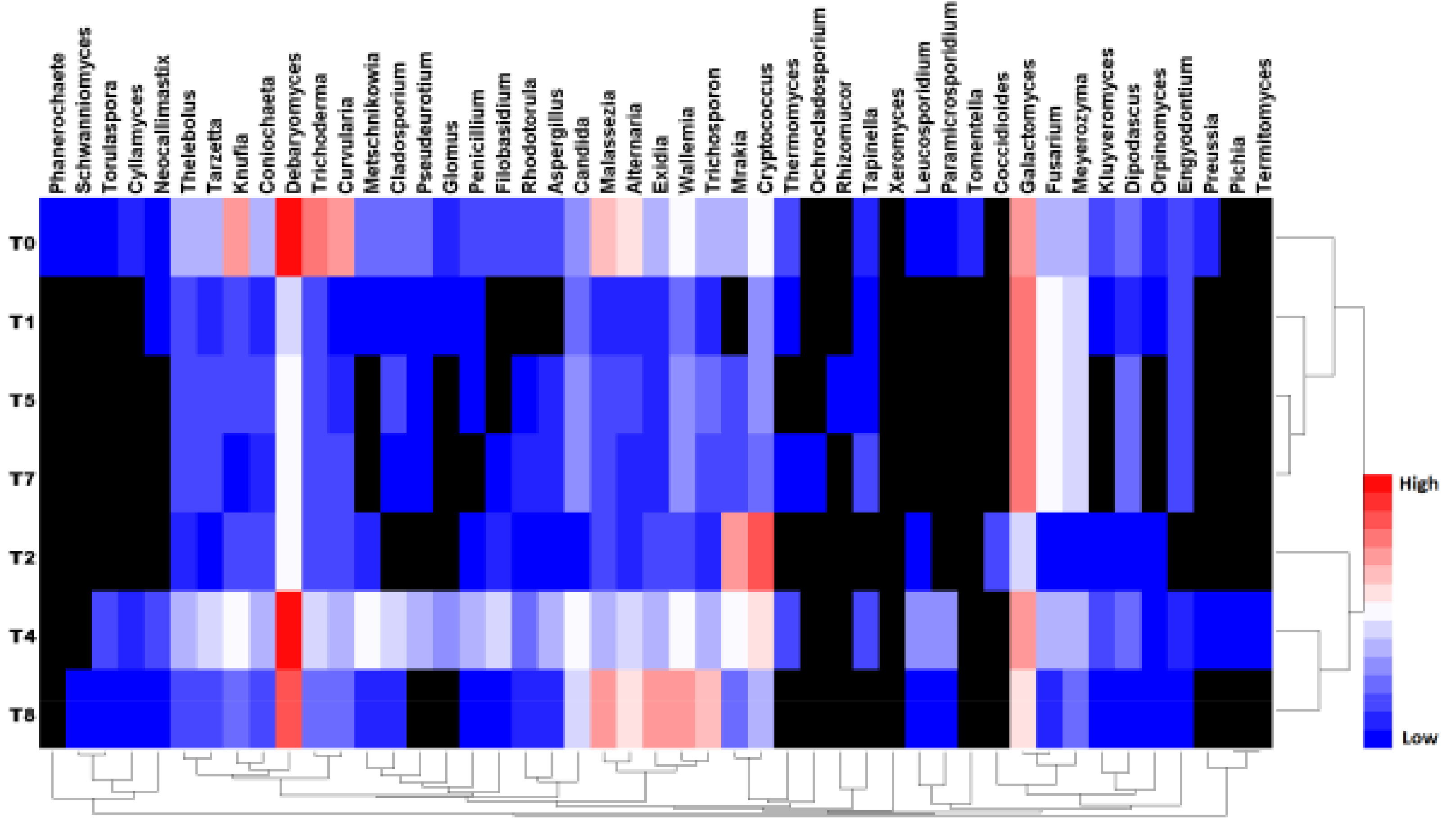


Figure 4.33 b : Heat maps analysis of fungal community structure generated from gut metagenomics of *L. rohita* based on clustering levels .

***T0 (Basal diet taken as control), T1 (*G. candidum* QAUGC01), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T2 (*E. faecium* QAUEF01), T4 (*B. cereus* QAUBC02), T8 (Commercial probiotic).**

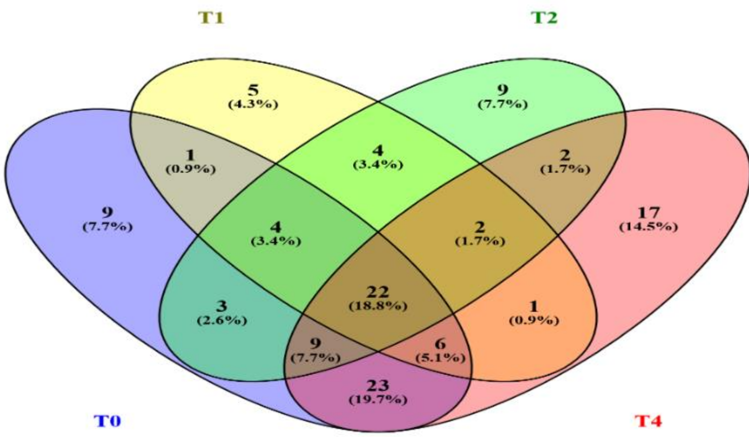


Figure 4. 34 a*:* **Venn diagram analysis of shared and unique bacterial genera in different groups of 90 day feeding trial in *L. rohita*. The values from the overlapping part represents the shared genera between groups, and the value from the nonoverlapping part represents the unique genera of that group**.

***T0 (Basal diet taken as control)), T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01) and T4 (*B. cereus* QAUBC02)**

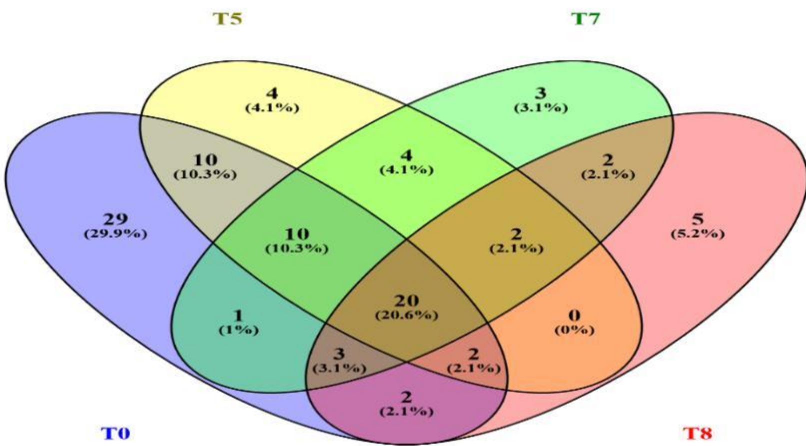


Figure 4.34 .b: Venn diagram analysis of shared and unique bacterial genera in different groups of 90 day feeding trial in *L. rohita* . The values from overlapping part represents the shared genera between groups, and the value from the nonoverlapping part represents the unique genera of that group.

***T0 (Basal diet taken as control), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T5 (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture) and T8 (Commercial probiotics).**

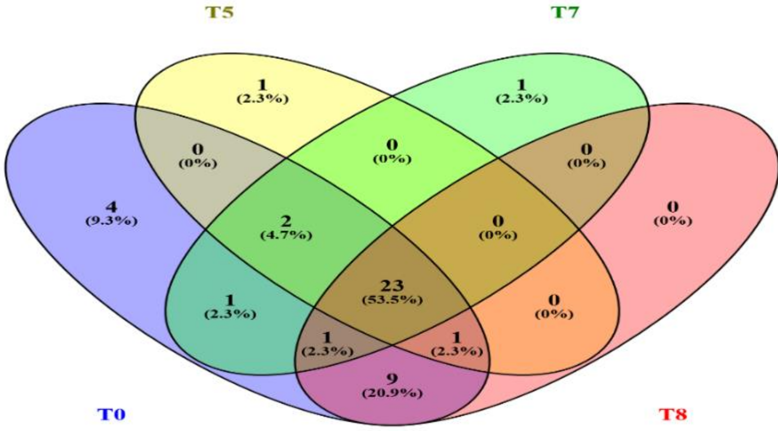


Figure 4. 35. a: Venn diagram analysis of shared and unique fungal genera of fish gut in different groups of 90 day feeding trial in *L. rohita* . The values from overlapping part represents the shared genera between groups, and the value from the nonoverlapping part represents the unique genera of that group.

***T0 (Basal diet taken as control), T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T5 (*G. candidum* QAUGC01 and *E. faecium*QAUEF01 co-culture) and T8 (Commercial probiotics)**

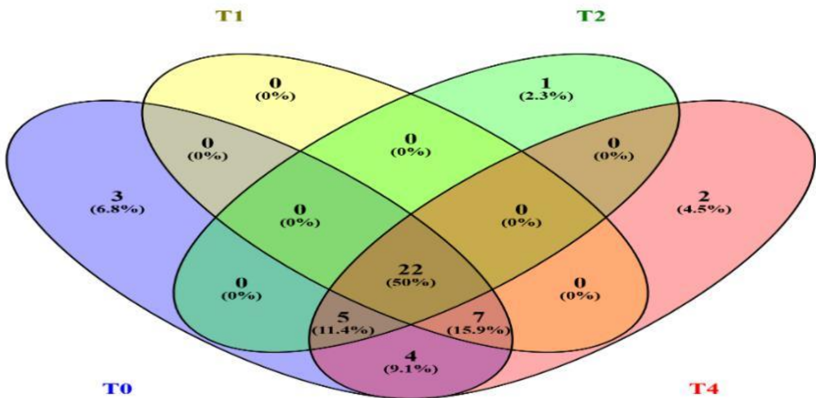


Figure 4.35 .b: Venn diagram analysis of shared and unique fungal genera of fish gut in different groups of 90 day feeding trial in *L. rohita* . The values from overlapping part represents the shared genera between groups, and the value from the nonoverlapping part represents the unique genera of that group.

***T0 (Basal diet taken as control)), T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01) and T4 (*B. cereus* QAUBC02).**

4.16 Proteomic analysis

A diverse group of proteins were expressed by the fishes fed on *G. candidum* QAUGC01 supplemented diet. The extracted proteins were categorized as regulatory proteins, stress proteins and carbohydrate metabolism proteins. Stress proteins help the cells to survive under harsh environmental conditions. A large number of proteins were found in present study involved in tricarboxylic acid cycle which is basically the oxidation of organic material to drive energy for cellular growth.Using MALDI TOF/TOF following peptides from *G.candidum* QAUGC01 were identified from lyophilized fish intestine mucosal sample (Table 4.17)

Table 4. 17: Proteomic analysis of *G. candidum* QAUGC01 from fish intestine mucosa

Accession number	Classification of proteins	Names of proteins	References
CDO56770.1	Regulatory protein	Mitochondrial malate dehydrogenase; catalyzes interconversion of malate and oxaloacetate; involved in the tricarboxylic acid (TCA) cycle; phosphorylated	(Reinders *et al.*, 2007)
CDO53410.1	Regulatory protein	Mitochondrial RNA polymerase; single subunit enzyme similar to those of T3 andT7 bacteriophages; requires a specificity subunit encoded by MTF1 for promoter recognition; Mtf1p interacts with and stabilizes the Rpo41p-promoter complex, enhancing DNA bending and melting to facilitate pre-initiation open	(Sanchez-Sandoval *et al.*, 2015)

		complex formation; Rpo41p also synthesizes RNA primers for mitochondrial DNA replication	
CDO51861.1	Regulatory protein	Subunit of RNA polymerase III transcription initiation factor complex; part of the TauB domain of TFIIIC that binds DNA at the BoxB promoter sites of tRNA and similar genes; cooperates with Tfc6p in DNA binding; largest of six subunits of the RNA polymerase III transcription initiation factor complex (TFIIIC)	(Geiduschek, 2001)
CDO56219.1	Regulatory protein	Second-largest subunit of RNA polymerase III; RNA polymerase III is responsible for the transcription of tRNA and 5S RNA genes, and other low molecular weight RNA.	(Xiao & Grove, 2009)
CDO53134.1	Regulatory protein	Positive regulator of the Gcn2p kinase activity; forms a complex with Gcn20p; proposed to stimulate Gcn2p activation by an uncharged tRNA	(Kubota *et al.*, 2001)

CDO54507.1	Carbohydrate metabolism	Fumarase; converts fumaric acid to L-malic acid in the TCA cycle; cytosolic and mitochondrial distribution determined by the N-terminal targeting sequence, protein conformation, and status of glyoxylate shunt; phosphorylated in mitochondria.	(Regev-Rudzki *et al.*, 2009)
CDO53482.1	Stress protein	Ribosomal 60S subunit protein L43B; homologous to mammalian ribosomal protein L37A, no bacterial homolog; RPL43B has a paralog, RPL43A, that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress.	(Ban *et al.*, 2014)
CDO57900.1	Regulatory protein	Sensor of mitochondrial dysfunction; regulates the subcellular location of Rtg1p and Rtg3p, transcriptional activators of the retrograde (RTG) and TOR pathways;	(Liu *et al.*, 2003)

		Rtg2p is inhibited by the phosphorylated form of Mks1p.	
CDO53083.1	Regulatory protein	Subunit of mitochondrial NAD (+)-dependent isocitrate dehydrogenase; complex catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle 2.	(Cupp & McAlister-Henn, 1992)
CDO52200.1	Protein synthesis	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S8, no bacterial homolog; RPS8A has a paralog, RPS8B, that arose from the whole genome duplication.	(Dieci *et al*., 2009)
CDO55896.1	Stress proteins	Thioredoxin peroxidase; acts as both ribosome-associated and free cytoplasmic antioxidant; self-associates toformhigh-molecular weight chaperone complex under oxidative stress; chaperone activity essential	(Lu *et al*., 2013)

		for growth in zinc deficiency; required for telomere length maintenance; binds and modulates Cdc19p activity; protein abundance increases, forms cytoplasmic foci during DNA replication stress; TSA1 has a paralog, TSA2, that arose from the whole genome duplication.	

Principal coordinate analysis was done among various factors which showed how various factors are co-evolving. Indigenous probiotics as use of T1 (G. *candidum* QAUGC01), T5 (G. *candidum* QAUGC01 and *E. faecium* QAUEF01) and T7 (G. *candidum* QAUGC01 and *B. cereus* QAUBC02) accompanied by increase in health promoting microflora which improved overall physiology whereas commercial probiotic use was accompanied by occurrence of pathogens (Figure 4.36).

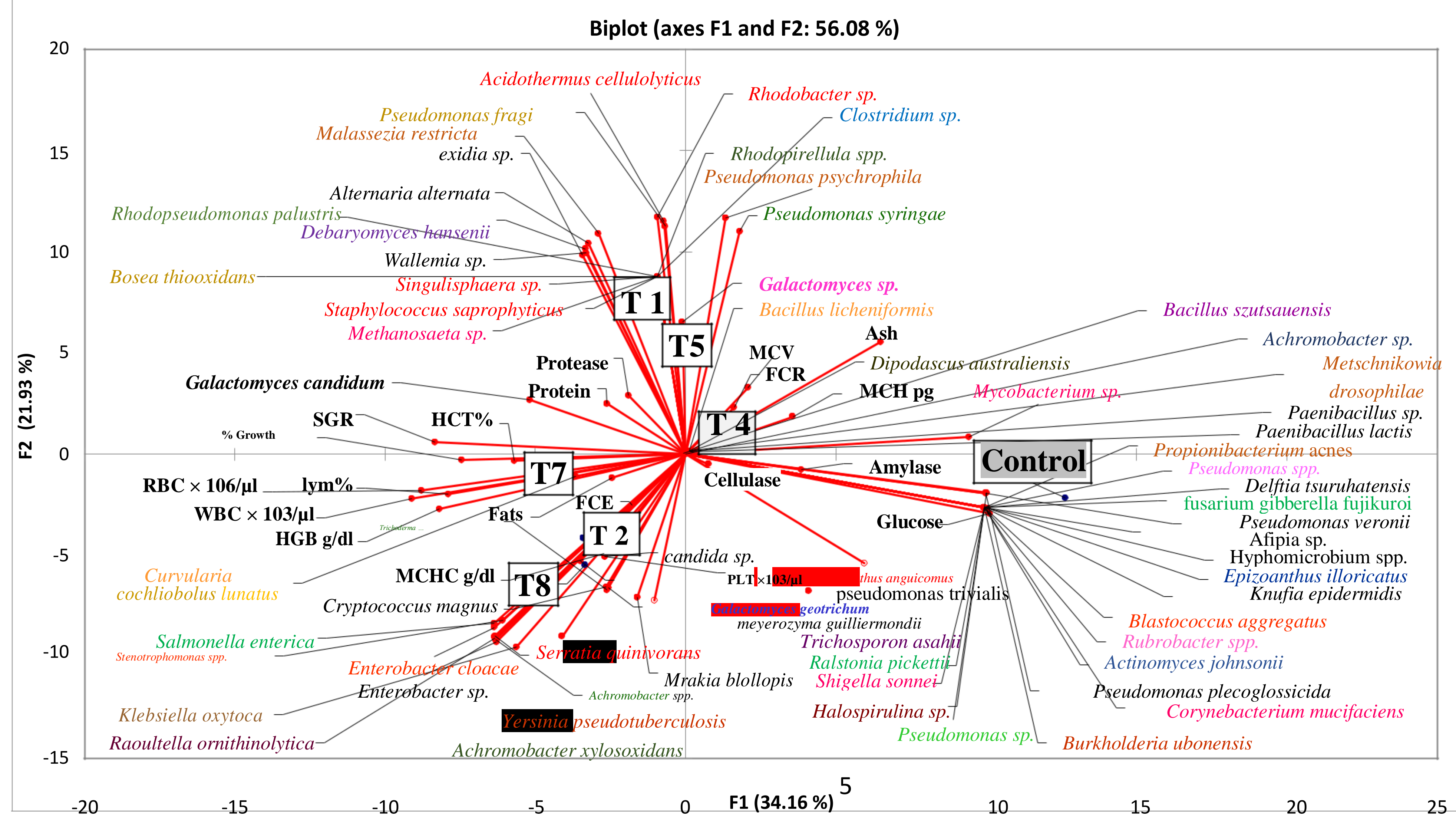


Figure 4. 36: Principal component analysis of correlation between factors

5. Discussion

5. Discussion

5.1 Phase–I Isolation, characterization and screening of strains for probiotic potential

The gut microbiome is crucial for the productivity and health of fish, it also protect host from various stress factors. Gut microbiome dysbiosis results in inefficient feed digestibility, compromised health status and low productivity. Moreover, considering side effects of conventional chemotherapeutics, probiotics seems to be an effective replacement provided; they remain viable in host gut (López *et al.*, 2003). Being non pathogenic probiotics have no bad impact on environment (Dawood & Koshio, 2016). It has been proven that most species of lactic acid bacteria are generally regarded as safe (GRAS). They help in mainting healthy gut microflora (Scourboutakos *et al.*, 2017). Lactic acid bacteria had been extensively applied in fish farming due to its promising health effects (Dawood *et al.*, 2016). The benefits imparted by probiotics depend on their interactions with gut microbial population and their ability to cope with digestive tract stresses. Microbial strains used in present study were isolated from local fermented milk product (Dahi), silage and fish gut. Apart from conventional sources of probiotics such as microbes from human gut, animal gut and the other sources used for probiotic microorganisms include GI tract of animals, fermented and non-fermented food sources, air and soil. Food microbiologists used food originated microbes as probiotics (Vinderola *et al.*, 2017; Zielińska & Kolożyn-Krajewska, 2018). Locally isolated probiotics were selected to understand the chance of higher performance as compare to the non-indigenous microbes because of the same ecological niches. It is suggested that while preparation of probiotics for animal use native isolates should be preffered for best out comes due to the same habitat and ecological adaptability (Markowiak & Śliżewska, 2017, 2018) . The probiotic intended to be used in animals are mostly isolated from the intestinal tract of its host (Benavides *et al.*, 2016; Interaminense *et al.*, 2018; Wanka *et al.*, 2018). Twelve bacterial isolates from fish gut were considered for identification and characterization. Eleven isolates were gram positive cocci while one was gram positive rod, all isolates were catalase and oxidase negative. The identified isolates from fish gut were *B. cereus* QAUBC02, *E. hirae* QAUF01, *E. faecium* QAUF18 and *E. mundtii* QAUF20. *Bacillus cereus* was also isolated

from intestinal tract of flatfish . The *B. cereus* has been isolated form marine fish guts of flatfish and *L. rohita* (Ghosh *et al.*, 2010; Wanka *et al.*, 2018). Previously lactic acid bacteria was also isolated from marine fishes gut included *Enterococcus* sp (Alonso *et al.*, 2019). Lactic acid bactria was also isolated from *Nile tilapia* (Bagunu *et al.*, 2018). *E. mundtii* has recently been reported rom Dahi as potential probioitcs (Nawaz *et al.*, 2019). In Total thirteen microbial isolates, one yeast and twelve bacterial were selected for *in-vitro* probiotic potential assessment. The *G. candidum* QAUGC01 and *E. faecium* QAUEF01 were of Dahi origin, *E. hirae* QAUEH01 was isolated from silage and *B. cereus* QAUBC02, *E. mundtii* QAUF20, *E. faecium* QAUF18, *E. hirrae* QAUF01 and 6 molecularly unidentified (Gram +ve cocci) were from fish gut. There is enough literature about application of fish feed supplementation with probiotics. Mohapatra *et al.* (2012) reported the use of *B. subtilis, S. cerevisiae* and *L. lactis* in *L. rohita*.

Isolates should tolerance acidity and should resistant to bile salt in order to ensure effective use as potential probiotics (Ayyash *et al.*, 2018). In the present study the isolates and co-culture preparations showed significant variations which might be due to the different mechanisms for acid and bile tolerance. Similar variations among isolates were observed earlier (Nami *et al.*, 2019). Stomach acidity is the first stress faced by probiotic microbiota in gastrointestinal tract. The comparatively highest survival at pH 2.0 and 5.0 was observed for *E. faecium* QAUEF01 followed by O14 F7, F19, O2 (unidentified fish gut isolates). Remaining isolates and combination of isolates didn’t show significant survival at pH 2. Some of the *Enterococcus* isolates from traditional dairy survived at low pH (Nami *et al.*, 2019). Previous studies showed that lactic acid bacteria can effectively tolerate acid and bile (Angmo *et al.*, 2016). Lactic acid bacterial isolated from raw and fermented milk showed varied response to acidic pH, bile and antibioitc sensitivity (Masalam *et al.*, 2018). A novel lactic acid bacterial strain *Lactobacillus plantarum* from fermented spider plant has bile salt hydrolase activity due to the presence of its gene in its plasmid (Yasiri *et al.*, 2018). Probiotics survival at low pH might be link with ATPase production gives microorganisms tolerance in acidic conditions and it had been reported to be produced by Lactic acid bacteria. It was found from the earlier studies that *E. faecium* isolated from the rhizosphere survived for 3 hours at pH 3 but could not tolerate pH 1.5-2 (Singhal *et al.*, 2019). The *E. faecium* showed good survival at pH 3 but at pH 2.5 survival

rates were moderate after 2 hours (Saelim *et al.*, 2012). *Enterococcus hirae* had been reported to produce the ATPase in large extent when grown in acidic condition (Kobayashi *et al.*, 1986). F0F1ATPase system helps the bacterial cells to survive in acidic stress by using ATP and translocation of protons from the cells through the membranous channels thus raising the intracellular pH (Sebald *et al.*, 1982). The knock out experiments revealed that the luxS gene was responsible for acid tolerance in *Lactobacillus* plantarum (Jia & Xie, 2018). Proton pumps, elevated expression of regulators, repairing proteins, regulatory proteins and alterations composition of membranes are few survival strategies adopted by cell during acid shocks (Cotter & Hill, 2003).

The bile salt in the proximal part of small intestine is another inhibitory factor encountered by the gut microbiota. In the bile salt media at 1.5g/L, pH 3 and lysozyme concentration was 100µg/ml were used. The was used as the fish gut pH varies from 2 to 5. After 2 hours incubation the maximum survival was recorded for *B. cereus* QAUBC02 (91.47±1.61%) followed by F19 (87.64±1.76%) and O1 (78.21±3.41%). The percentage survival showed by *G. candidum* QAUGC01 (38±0.576%), *E. faecium* QAUEF01 (54.36±1.94%) and *E. hirae* QAUEH01 (54.23± 4.95%) percent survival respectively. The coculture of *B. cereus* QAUBC02 and *G. candidum* QAUGC01 and *G. candidum* QAUGC01 and *E. faecium* QAUEF01 showed good survival after 2 hours. After 6 hours of incubation *B. cereus* QAUBC02 consistently showed higher survival (87.31±1.27). The other single strains that showed more than 50% survival rate were *E. faecium* QAUEF01 and O1, O2 and F7. The survival percentage of *G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02 combination was (88.21±1.49), the percentage survival of coculture of *E. faecium* QAUEF01 *and G. candidum* QAUGC01 was (69.34±2.98)%. After 24 hours *B. cereus* QAUBC02 percentage survival rate was (85.01±1.85)%. The other single strains that showed more than 50% survival in bile were F19, F7, O1 and F8. The co-culture of *G. candidum* QAUGC01 *and B. cereus* QAUBC02 showed (79±2.13) % percent survival rate. Comparative evaluation of single strain versus coculture showed that the coculture of bacterial and fungal strains efficiently resist the bile stress. This might be due to synergistic association of bacteria and fungus. The interactions between fungus and bacteria exist in almost every ecosystem. These are also very curcial for health of animals and plants (Deveau *et al.*, 2018). These interactions varied from antagonism to mutualism that effect

the growth, reproduction, transport/movement, nutrition, stress resistance and pathogenicity of each other (Deveau *et al.*, 2018). Numerous biotechnological applications are attributed to the by-products of fungal and bacterial interactions. There might be metabolic interaction between fungus and bacterial in which they are inter-dependent on each other. In certain cheeses the lactic acid is metabolized by the yeasts like *Geotrichum candidum* and *Debaryomyces hansenii* and the later on deacidified surface becomes conductive for the growth of aerobic bacterial (Mounier *et al.*, 2005). Such properties were previously recognized for *G. candidum* (Boutroua & Gueguen, 2005; Khoramnia *et al.*, 2013).

The other isolates (O1 and F7) from fish gut that showed more than 50% survival rate in presence of bile after 2hrs, 6hrs and 24 hrs were found to be O1 and F7. Our results were in accordance to the study conducted by Tenea *et al,* 2016, it was reported that probiotic isolates from fish gut showed >90% survival at pH 2.5 to 4.5 and 0.3% bile salt concentration (Benavides *et al.*, 2016; Tenea, 2016). Although percentage survival of isolates in current study was comparatively low, that could be due to different reasons. The endurance to bile salts by certain microorganisms suggests particular mechanisms which prevent lysis by bilayer disintegration. The concentration of the bile excretion depends on various factors such as feed type and age of animal (Begley *et al.*, 2005). Yeast and bacteria display variety of survival mechanisms to combat bile stress such as metabolism of bile, production of bile salt hydrolase enzymes (BSH), modifications in the surface molecules and efflux system (Soccol *et al.*, 2010).

In our study *E. faecium* QAUEF01 and *E. hirae* QAUEH01 both survive well after exposure to bile salt media for six hours. Lactic acid bacteria encode several bile salt hydrolase (BSH) encoding genes that might enable them to successfully survive in gastrointestinal tract (Dicks & Botes, 2009). In our study, the co-culture of yeast and bacteria showed better survival as compared to single strains this might be due to the complementary pathways by mixed cultures resulting in better tolerance. Bile salt hydrolase of microorganisms depends on various factors such as diet composition, interaction with resident microbial community and response to acid and bile stresses (Succi *et al.*, 2005). Survival and colonization in small intestine is more important to impart

positive impacts on host rather than acid tolerance as owing to introduction of new approaches the acid sensitive strains can be buffered in stomach (Huang & Adams, 2004). Probiotic should have ability to adhere with the intestinal epithelium to colonize and sustain well in the gut (Bernet *et al.*, 1993). This potential characteristic was assessed by measuring the cell surface hydrophobicity of isolates towards xylene (Aswathy *et al.*, 2008; Sakandar *et al.*, 2018). The total number of micro-organisms attached to hydrocarbon layer depicts the colonization ability of isolate. The wide variations (11±1.74-40.35±3.45)% found in the isolates and coulture of isolates used in the study. Our results are in according with the previous study done on different strains of lactic acid bacteria showing range between 8.37-70.76% (Puniya *et al.*, 2012). In present study comparative evualtion of hydrophobicity showed the single strains that showed maximum acitivity was *B. cereus* QAUBC02 (30.89±2.52) percent, *G. candidum* QAUGC01 (21.96±1.78), *E. hirae* QAUEH01 (17.29±1.59), *E. faecium* QAUEF01 (15.91±2.39) and the least hydrophobicity was shown by *E. mundtii* QAUF20 (5.14±1.57). The maximum hydrophobicity observed in co-culture of *G. candidum* QAUGC01 and *E. faecium* QAUEF01 was (40.35±3.45) % followed by coculture of *G. candidum* QAUGC01 and *E. hirae* QAUEH01 (27.28±1.29) %. The variations observed in hydrophocity might be due to the differences in level of expression of surface proteins or due to different environmental factors that effect the degree of expression (Kaushik *et al.*, 2009). Hydrophobicity depends upon many factors such as physiological state of cell, media in which cells are present and surface attached proteins might also be participate in determination of hydrophobicity (Nwanyanwu *et al.*, 2012). Better hydrophobicity is associated with presence of proteinaceous substance present on the surface of cell (Pelletier *et al.*, 1997). It was demonstrated in previous studies that 92.9 % of *E. faecium* strains displayed very low adhesion competency (Wijaya *et al.*, 2003.). It was reported in another study that 79.2% of the tested *E. faecium* strains have low hydrophobicity activity i.e. below 30% similar results were found in our study in which most of the isolates and combination of yeast and bacteria hydrophobicity was found to be less than 30% (Bhardwaj *et al.*, 2010).

Surface layer proteins attached to *L. acidophilus* M92 are thought to involve in its persistence in gastrointestinal tract thereby protecting the host from pathogens (Frece *et al.*, 2005). It was deduced that when the surface protein was damaged or removed high

reduction in its attachment to epithelial intestinal cells was observed (Xue *et al.*, 2013). Therefore, it is concluded that numerous factors along with hydrophobicity are involved in adhesion. It was found that *Lactobacillus rhamnosus* GG and *Lactococcus lactis* TIL448 was facilitated by pili the same structure help the pathogens to adhere the host (Meyrand *et al.*, 2013), similarly tight adherence pili and sortase dependent pili mediate the attachment of *B. breve* UCC2003 and *B. bifidum* PRL2010 with intestinal epithelium (Motherway *et al.*, 2011; Turroni *et al.*, 2013).

In our study, it was observed that all the isolates were sensitive to Vancomycin (VA30), and Chloramphenicol (C30). Earlier lactic acid bacterial strains were found to be sensitive to chloramphenicol (Klare *et al.*, 2007). Profiling of European probiotics showed multiple resistance patterns against antibiotics. During the present study, it was observed that our strain used in feed trial were safe as it was sensitive to Vancomycin. *Enterococci* isolated from farm animals showed resistance to vancomycin which were cured from unconventional antibiotic therapy and these resistance was due to the presence of antibiotic resistant genes such as *van*A, *van*B, *van*C1, *van*C2, *van*C3, *van*D and *vane* (Leclercq, 1997). Lactic acid bacteria showed resistance to numerous antibiotics and this resistance is most often non-transferable. This intrinsic resistance offers no safety concerns as these lactic acid bacteria are mostly sensitive to clinically used antibiotics when the incidences of opportunistic infections occur. Intrinsic resistance occurs to the Cephalosporins, β-lactams, Sulphonamides, and low levels of clindamycin and aminoglycosides (Leclercq, 1997). Variable resistance was also found among potential probiotic strains used in present study, *B. cereus* QAUBC02 was only resistant against cefpirome, ampicillin and piperacillin and *E. faecium* QAUF18 and *E. mundtii* QAUF20. Similar results were found by *Lactobacillus* strains from fruit processing by products which showed varied sensitivity against the used antibiotics (Garcia *et al.*, 2018).

Anti-pathogenic acitivity of potential probiotics is the another factor that imparts positive impact on the host. In the present study *B. cereus* QAUBC02 was found to be active against *L. monocytogenes* ATCC13932 and *S. aureus* ATCC 2593 that might be due to production of antimicrobial compound Cerein. It was deduced from the earlier studies that Cerein antibiotic produced by *Bacillus cereus* Bc7 was active against Gram positive bacteria (Oscáriz *et al.*, 1999). *E. faecium* QAUEF01 also inhibited the tested pathogens in the

present study. Previously it was found that the *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA produced antilisterial activity (Van Zyl *et al.*, 2019). In present study *G. candidum* QAUGC01 produced largest inhibition zones among all tested potential probiotics. It inhibited the growth of *S. aureus* (ATCC 2593)*, S. enterica* (ATCC14028) *and E. coli* (ATCC25922) giving zone of (30.66mm), (29mm) and (28.6mm) respectively. *Geotrichum candidum* was known to produce a variety of antimicrobial compounds some of them are phenyl lactic acid and indole acetic acid etc (S. Naz *et al.*, 2013). Anti-Listeria compounds were purified from *G. candidum* QAUGC01 and the two inhibitory compounds detected by numerous techniques were D-3-phenyllactic acid and D-3-indollactic acid (Dieuleveux, 1998s). *G. candidum* QAUGC01 has the ability to inhibit the growth of Gram positive, Gram negative bacteria and fungi (Gueguen *et al.*, 1974) similar results are found in our study by *G. candidum* QAUGC01.

Maximum zone of inhibition against *L.* monocytogenes (ATCC49594) was observed by combination of *G. candidum* QAUGC01 and *E. hirae* QAUEH01. *G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture showed highest antagonistic activity against *P. aeruginosa* (ATCC 27853). The results suggest that combination of yeast and bacterial strains produce diverse antimicrobial compounds rather than mono-strain probiotic. Earlier in a study a 1:1 mixture of two *Lactobacillus* strains namely SYNBIO® gave different levels of antagonistic activity against test pathogens. The antagonistic attribute (Coman *et al.*, 2014) . Multiple specie probiotic preparation consisting of specie belonging to more than one genus found to be more effective. The VSL#3 is a multispecies probiotic which is found to be more effective in treating pouchitis and ulcerative colitis as compared to convention antibiotic treatment and mono-strain probioitcs (Shibolet *et al.*, 2002).

Different combination of strains must be evaluated by *in vitro* testing in order to confirm that they should not produce inhibitory compounds for each other like bacteriocins, once it is confirmed that they are mutually beneficial towards one another they should be formulated into probiotics preparation for desired clinical benefits (Kailasapathy & Chin, 2000). The positive interrelationship between strains in probiotic mixture termed as proto-cooperation help to exchange growth factors such as amino acids, peptides, formate and CO_2 this synergistic effect often makes the combination of probiotics as effective for

enhancing growth and metabolism (Driessen *et al.*, 1982; Gomes *et al.*, 1998; Sodini *et al.*, 2000).

5.2 Phase –II Application of selected probiotics in *L. rohita*

The strains performed well in *in vitro* characterization were applied in second phase of study. A 90-days experimental trial was executed based on the assumption that strains having probiotic potential have a high probability of persistence in fish intestine and will ultimately enhance growth and health of host. Physiological parameters including growth, hematological parameters, enzymes and nutritional composition analysis were studied at 45th and 90th day of experiment.

After 45th day growth pattern showed that the weight gain (WG) of T7 fish fed on combination of *G. candidum* QAUGC01 and *B. cereus* QAUBC02 diet was significantly higher (P<0.05) than all other groups, while significantly lower WG was observed in group of fish fed basal diet same trend was shown by T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02)141.48±1.05 at 90th day (P<0.05) . The growth was significantly low (P< 0.05) for control. T0 (Control) and T4 (*B. cereus* QAUBC02) which vary non significantly with respect to each other while all other treatments showed significant impact on fish growth. T7 fed on diet supplemented with (QAUGC01 and QAUBC02 co-culture) showed the highest % weight gain of (123.30±2.79) which was 75.98% more that the control group. A previous study showed that fish fed brewer's yeast and lactic acid bacteria diets grew significantly (P < 0.05) faster than those fed a control (Dhanaraj *et al.*, 2010). African catfish (*Clarias gariepinu*) when fed with *Lactobacillus acidophilus* showed improved growth as compared to control (Al-Dohail *et al.*, 2009). Previous studies accredited that probiotic added feeds promoted gain weight in aquatic animals by triggering enzymatic activity thus facilitating digestion and maintaining health (Zhang *et al.*, 2010). There was report of 80% increased weight gain by adding *Bacillus coagulans* as the feed additive in the white shrimp in comparison to the basal fed control (Wang *et al.*, 2012). Increased body weight gain in *L. rohita* was observed by supplementing the feed with *B. circulans* as a potential probiotic (Ghosh *et al.*, 2003). Similarly increase in weight, FCR and SGR was observed in Mozambique tilapia when fed on probiotics in a four week feeding trial validating many previous studies (Gobi *et al.*, 2018). It has been reported that positive impact of probiotics on growth by supplemented yeast and *Bacillus* in fish that ultimately

enhance growth rate may be linked to stress reduction, increased appetite, and vitamin production, detoxification of feed ingredients, better intake and utilization of feed. It was reported that the growth depends on various factors including efficiency of digestive system, quality of water and rearing conditions collectively (De Silva & Anderson, 1994; Irianto & Austin, 2002b).

The specific growth rates (SGR) show a similar trend, significantly higher (P<0.05) value was observed with T7 (*G. candidum* QAUGC01 with *B. cereus* QAUBC02) giving value of i.e. (2.67±0.04) at 45th day while lowest result was obtained with basal diet (1.29±0.07). Specific growth rate showed a similar trend as growth rate at 90th day of experiment, T7 treatment (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) showed significantly higher value (1.0931±0.05), while lowest SGR was observed for basal diet (0.7610±0.066) (P<0.05).

An increase in SGR in comparison with the basal diet control in *L. rohita* was reported when fed a diet having a combination of probiotic microorganisms (Mohapatra *et al.*, 2012). Moreover, high SGR in *O. niloticus* fingerlings was observed when fed diet supplemented with probiotics (Biogen®) as compared to the control diet was observed . Significant increase in growth performance, growth and SGR are in agreement with previous studies conducted by (Allameh *et al.*, 2016; Dhanaraj *et al.*, 2010; Huang *et al.*, 2015; Mohapatra *et al.*, 2012). These studies proved that the probiotic added diets result in better growth and weight gain in carps. *Bacillus subtilis*, *Lactococcus lactis* and *S. cerevisiae* augmented diet with 10^{11} CFU/kg count could significantly improve SGR and weight gain in *L. rohita* (Mohapatra *et al.*, 2012). The increase in weight gain and improve SGR is linked with low FCR and high FCE. All of the potential probiotics alone or in combination considerably improved the FCR values. The FCR values in *L. rohita* in response to T1 (*G. candidum* QAUGC01), T2 (*E. faecium* QAUEF01), T3 (*E. hirae* QAUEH01) and T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) diets were (2.37±0.19), (2.3±0.13), (2.56±0.10), (2.29±0.07) respectively, significantly improved (P < 0.05). While significantly higher (P<0.05) feed conversion efficiency percentage (FCE %) was observed in T7 treatment fed on (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) (30.72±0.58). On the other hand, significantly lower FCE value was observed with basal diet (16.01±0.13) as compared to all other probiotic

treatments. The observations recorded at 90th day of feeding trial showed that feeding on potential probiotics in single or consortium form significantly improved the FCR and FCE. The highest FCR was (5.8279±0.15) in T4 *B. cereus* QAUBC02 fed group, while lowest FCR was noted in T2 (*E. faecium* QAUEF01) (5.02± 0.05). The FCE% of T2 (*E. faecium* QAUEF01) (19.92±0.199) and T7 (co-culture of *G. candidum* QAUGC01 and *B. cereus* QAUBC02) (19.38±0.033) was significantly higher than T0 basal diet at the 90th day of feeding trial.

The positive impacts on growth parameters in *O. niloticus* can be realted with the supplementation of commercially available probiotics Pronifer® or Biogen®. This can increase cell metabolism, energy of the cell, raise the feed utilization capacity (Abdelhamid *et al.*, 2002; Eid & Mohamed, 2008). The use of *Lactobacillus acidophilus* as a probiotic in snake head (*Channa striata*) resulted in improved growth performance in terms of better feed utilization and enhanced survival rate (Talpur *et al.*, 2014). Moreover our results are in agreement with an earlier studies in Nile tilapia when *S. cerevisiae* and *E. faecium* enriched diets were administered as feed supplement improved growth parametes (Lara-Flores *et al.*, 2003). Probiotics help in feed conversion efficiency and live weight gain (Sáenz de Rodrigáñez *et al.*, 2009). Additionally, all the probiotic-supplemented diets resulted in growth to be higher than that of the control diets, suggesting that the addition of probiotics mitigated the effects of the stress factors. This resulted in better *O. niloticus* performance, with better growth parameters in the diets supplemented with the yeast (Lara-Flores *et al.*, 2010) . The positive results of the tested probiotics in present study and those from previous studies suggest that the raise in weight gain and improve feed utilization might be due to stimulation of appetite, improved nutrition, enhance nutrient digestibility and digestive enzyme activation by those applied probiotics (Irianto & Austin, 2002a; Waché *et al.*, 2006). A study based on the application of *G. candidum* QAUGC01 as a probiotic in juvenile Rohu confirmed that it enhanced growth, FCR, crude protein and intestinal enzymes as compared to fry fed on basal diet. Thus probiotic supplemented diet can compensate digestive enzyme deficiency specially in early rearing stage when fishes have inefficient digestive tract (Ibrar *et al.*, 2017). The enzyme potential of *G. candidum* QAUGC01 is validated by many published reports (Bakar, 2014; Muhammad *et al.*, 2019) .

Hematological parameters are important index that depicts fish health status and for ichthyologist they are important tool for finding of fish disease and pathological condition (Ayoola *et al.*, 2013). Hematology of fish is variable with respect to age, size, type of nutrition, feed supplements such as probiotics (Osuigwe *et al.*, 2005). The present study showed that all the tested probiotics had impact on blood parameters as compared to basal diet taken as control. The T7 (*G. candidum* QAUGC01 *and B. cereus* QAUBC02 co-culture) treatment had a highly significant effect ($P<0.05$) on RBC (2.43±0.023) $10^6 \mu L^{-1}$. count, hemoglobin (g dl^{-1}) concentration (9.7±0.35) and hematocrit percentage both at 45th day The treatment (T2) *E. faecium* QAUEF01 also significantly higher ($P<0.05$) RBCs count (2.53±0.029) $10^6 \mu L^{-1}$ at 45th day of trial. Our data present a significant higher level Hematocrit in *L. rohita* group fed on (T4) *B. cereus* QAUBC02 enriched diet as compared to control group fed basal diet at 45th day. These findings are in agreement with the earlier study in which an increase in RBC, HGB and HCT values in *Catla Catla* fed probiotic (*L. acidophilus*) was observed as compared to control group (Renuka *et al.*, 2014). Likewise an increase HGB value of a probiotic fed groups in our results is in agreement with the earlier findings (Capkin & Altinok, 2009). The data obtained at 90th day of the trial showed that fish fed with *G.candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture (T7), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture (T6), *B. cereus* QAUGC02 (T4) and commercial probiotic (T8) showed higher RBCs count and HGB level which was significantly ($P<0.05$) higher than T0 fed with basal diet. Highest RBCs count was in T7 (*G. candidum* QAUGC01 and *B.cereus* QAUBC02 co-culture) was (2.7533±0.008) $10^6 \mu L^{-1}$. Same trend was observed in HGB concentration. T7 (*G. candidum* QAUGC01 and *B.cereus* QAUBC02 co-culture) showed maximum hemoglobin concentration was (11.533±0.29) gdL^{-1}which was significantly ($P<0.05$) higher than T0 (4.6000±0.34) gdL^{-1}. HCT count of groups fed with probiotics was significantly higher than fish fed with basal diet. Highest HCT value (39.1333±0.72) %was noted in T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) and lowest (13.2667± 0.69) in basal diet T0. In present study different probiotic treatments improved MCH and MCHC value significantly to different levels as compared to control both at 45th and 90th day of the experimental trial. T3 (*E. hirae* QAUEH01) fed group showed significantly high MCH (43.9± 0.58)pg at 45th day and T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) showed

significantly high MCH (42.80±0.83)pg at 90th day of experimental trail. The significantly improved MCHC (53.9±0.58)g/dl^{-1} at 45th day was observed by T3 (*E. hirae* QAUEH01), whereas the significantly high value of MCHC (64.93 ± 0.93) g/dl^{-1}(Ayoola *et al.*, 2013). In the current study, probiotics (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture) fed group (T7) have 22.58% more WBC count as compared to control group of fish fed basal diet at 45th day of experiment. The significant increase in WBCs might be due to hematopoietic stimulation by probiotics also reported higher WBC count in probiotic treated group as compared to control group which is similar to the results obtained in present study (Hassaan *et al.*, 2014). In our study fish fed with probiotics supplemented diet T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture), T8 (commercial probiotics), T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01) resulted significantly higher count of WBC as compared to the control fed with basal diet while there was no significant difference between them. These results are in agreement with the results reported previously (Harikrishnan *et al.*, 2010b). Lymphocyte count at 45th day showed no statistically significant difference between all the treatments. Highest lymphocytes count at 90th day was observed in *B. cereus* QAUBC02 (T4) (97.8667±0.95) and lowest (77.6667±0.52) in control group. Maximum platelet count was observed by commercial probiotic T8 (77±0.57)10^{3}µl^{-1} at 45th day and maximum platelet count (387.44±1.45)10^{3}µl^{-1} at 90th day of probiotic feeding trial was again recorded for commercial probiotic (T8). Platelets count and lymphocytes count in probiotics fed groups was also significantly higher as compare to the control group fed on basal diet. But in case of platelets count some treated groups showed lower count as compare to the control. Our results were in agreement with the previous studies that the platelets and lymphocytes count can be affected by probiotic treated diet (Marzouk *et al.*, 2008).

In the present study at 45/90 day of experimental trial, protease activity in *L. rohita* fed on (T5) co-culture of *G. candidum* QAUGC01 and *E. faecium* QAUEF01 was significantly higher ($P < 0.05$) followed by T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) as compared to control group of fish fed basal diet both at 45 and 90 day respectively. A raise in feed conversion efficiency in the fishes fed on T2 (*E. faecium* QAUEF01) and T5 (co-culutre of and *E. faecium* QAUEF01) was observed which correlated with enzymatic potential. These enzymes also make the digestion process

efficient by enhancing metabolism. (Gildberg *et al.*, 1997; Silva *et al.*, 1995). The synthesis of nutrients and enzymes by probiotics increases the digestive efficiency (Wang, 2007; Ziaei-Nejad *et al.*, 2006). *G. candidum* QAUGC01 is known for its protease activity is considered as best candidate for commercial use in industry (Bakar, 2014; Muhammad *et al.*, 2019). *Enterococcus faecium* E745 and *Enterococcus faecium* PSB 5 isolated from protein rich soils had immense protease potential (Boukhtache *et al.*, 2017). It is assumed that the probiotic after passing through the stomach of fish, use sugar for their growth, multiply in the intestine and produce protease enzyme, which increase digestion of feed ingredients (El-Haroun *et al.*, 2006). It was reported in an earlier study that higher Like our results protease activities in *Litopenaeus vannamei* fed probiotic (*B. coagulans*) enriched diet was obsereved as compared to basal fed diet . While maximum amylase and cellulase activity was showed by *B. cereus* QAUBC02 (T4) both at 45th day and 90th day respectively which was significantly higher as compared to control. *Bacillus* sp. are the key source for microbial enzymes at commercial level (Ray *et al.*, 2010). Study based on *in-vitro* analysis showed that *B. circulans* Lr 1.1, *B. pumilus* Lr 1.2 and *B. cereus* Lr 2.2, isolated form the gut of rohu fingerlings produce proteolytic enzymes appreciably (Ghosh *et al.*, 2002; Gupta *et al.*, 2002; Liu *et al.*, 2009). It was reported that the presence of amylolytic bacteria *Bacillus circulans*, *Bacillus pumulus* and *Bacillus cereus* from the gut of *Rohu* which indicates the possible link between enzyme produced and feeding pattern (Ghosh *et al.*, 2002). Amylase is also a key enzyme which is responsible for the digestion of carbohydrates. It was observed in an earlier study that the shrimps (*Penaeus vannamei*) showed significantly ($P<0.05$) higher amylase activities when fed on probiotics as compared to group fed on basal diet. (Wang, 2007; Zhou *et al.*, 2009) It is explained in a previous study that fish secrete their extracellular enzymes with respect to their dietary habits (Deobagkar *et al.*, 2012). It was confirmed that the GIT of *L. rohita* being rich in cellulases might be due its herbivorous feeding habits (Kar & Ghosh, 2008). Previous studies detected significantly enhanced levels of cellulase activity in shrimp (*Penaeus vannamei*) when the diet was supplemented with probiotics as compared to control (Wang, 2007). The GI tract of *L. rohita* is predominantly populated with amylolytic and cellulolytic bacteria as compared to proteolytic ones (Ghosh *et al.*, 2010). A recently published report has confirmed the presence of extracellular enzymes producing *Bacillus* sp from GIT of

L. calbasu (Kavitha *et al.*, 2018). It was investigated that certain species of Indian carps that were efficient cellulase producers (Ray *et al.*, 2010). An extracellular protease was excreted by *B. cereus* in the intestinal tract of brackish water fish (Esakkiraj *et al.*, 2009).

The chemical composition analysis of dry flesh mass of *L. rohita* at 45th day reared on QAUGC01 and QAUEH01 co-culture (T6) supplemented diet showed significantly higher ($P<0.05$) crude protein content (74.38±0.17%) as compared to all other groups. In the present research work, the *G. candidum* QAUGC01 (T1), *E. faecium* QAUEF01 (T2), *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture (T6) and *G. candidum* QAUGC01 and *B. cereus* QAUBC02 (T7) supplemented feed had a significant effect on the proximate composition of crude proteins while at 90 day it was observed that in T3 (*E. hirae* QAUEH01) has significantly higher crude protein content as compared to all other treatments used. An earlier investigatioin suggested that Brewer's yeast, *S. cervisiae* being rich in protein provides amino acids and vitamins to animal feed. It was reported the diets containining *Lactobacillus coagulans* and *S. cervisiae as* probiotics can provide protein to the host(Swain *et al.*, 1996) . The crude fat content of *L. rohita* was significantly higher in T6 *G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture) at 45 day of experimental trial while T1 (*G. candidum* QAUGC01) and T8 (commercial probiotic) treatment showed significantly high crude fat content. Numerous investigations have confirmed the raised protein and fat contents of fish carcass in response to probiotics suplemneted diets might due to better nutrient utilization (Hassaan *et al.*, 2014; Lara-Flores *et al.*, 2003; Noveirian, 2012). Based on previous and current study results it is suggested that the changes in the chemical composition of fishes after fed with probiotics might be due to the variation in the accumulation rate and formation of muscles, improved feed consumption, better digestibility of the nutrients and active absorption (Abdel-Tawwab *et al.*, 2008; Rumsey *et al.*, 1990; Soivio *et al.*, 1989).

Glucose is an indicative of stress response though it is not very reliable source of stress evaluation (Flodmark *et al.*, 2002; Mommsen *et al.*, 1999). Probiotic fed fishes showed less blood glucose level as compared to control fed fishes this might be due to fact that probiotics alleviate stress thus lowering glucose level. The present results are supported by the previous study concluding that the probiotics fed groups have low glucose levels as compared to control (Mohapatra *et al.*, 2014). In the present research glucose level was

significantly decreased in groups fed the combination of yeast and bacteria as potential probiotic in *L. rohita* i.e. (*G. candidum* QAUGC01 and *B. cereus* QAUBC02) (T7) and (*G. candidum* QAUGC01 and *E. hirae* QAUEH01) (T6) supplemented diet as compared to control group. At day 90 lowest value was recorded in T6 (*G. candidum* QAUGC01 and *E. hirae* QAUEH01 co-culture) (45.23±1.12) and T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture (45.96±0.14).

Plasma glucose is one of the stress indicators in fish (Menezes *et al.*, 2006). Change in physiology of fish might cause a variation in serum glucose levels. Investigation concerning constancy and fluctuation in glucose serum levels can be linked with high plasma cortisol thus explaining the variation as observed in current study in glucose levels might be due to the inadequate ingestion of probiotics in different groups (Mommsen *et al.*, 1999).

The recommended range for hematocrit in fishes is 20-35% which rarely exceeds 50% (Clark *et al.*, 1979). The hematocrit value for most of the treatments used in present study lie within the normal range. Slight variation observed in current study may be due to varaitions in species, diet, environmental factors, age and size of the fish respectively. Probiotic fed groups showed obvious increase in blood parameters our study. This might be due to ehnhanced immune responses in probiotic supplemented treatments as suggested by (Panigrahi *et al.*, 2005a). This also supports that fact that probiotic supplemented diet keep fishes healthier (Gabriel *et al.*, 2004). There were slight variations with respect to MCH (27-31 pg $cell^{-1}$) our range was from 32-42. Our data showed the most of the treatments fall in normal range of MCHC (32-36 gdL^{-1}), some of the treatments as showed slight variations , as far as MCH is concerned some of the treatments used in our data was in recommended range (80-100 fl/cell) but fluctuations did exist due to numerous factors (George-Gay & Parker, 2003).

Correlation among physiological variables of the study (45th and 90th day).

Different physiological parameters were studied and relationship between different variables were determined by Pearson correlation at significant level alpha 0.05 ($P<0.05$) (Table 4.12a and 4.12b). WBCs showed significant positive correlation with RBCs, HGB, PLT, % growth, SGR, protein whereas negative correlation with protease and glucose RBCs showed significant positive correlation with WBCs, HGB, HCT and protein and

significant negative correlation with MCH and MCHC while at 90th day white blood cells (WBC) showed significant positive relationship with RBC, HGB, HCT, MCHC, PLT, LYM%, percentage growth rate and specific growth rate while significant negative relationship with MCV and MCH. At the 90th day of trial relationship of RBCs with other parameters was non-significant. A significant positive relationship was found between Red blood cells (RBC) and HGB, HCT, LYM%, percentage growth rate and specific growth rate. Red blood cells showed significantly negative relationship with MCH while with other parameters red blood cells relationship was non-significant. In a previously published correlation study based on hematological parameters a strongly positive correlation was observed between RBCs and HCT, a moderately positive correlation was observed between RBC and HGB and a significantly negative correlation was observed, a negative correlation was observed between RBC and MCH and RBC and MCV (Goda, 2008). HGB showed a positive significant trend with WBCs, RBCs, % growth, FCE, SGR and protein and negative significant correlation was found out with glucose at 45th day while at 90th day hemoglobin showed significant positive relationship with HCT, LYM%, percentage growth rate and specific growth rate while relationship with other parameters was non-significant. Earlier it was reported that the probiotics supplementation with Bacillus (*Bacillus licheniformis* and *Bacillus subtilis*) in Asian sea bass diets significantly improved growth, FCR, SGR, RBCs, WBCs, HGB, body composition and enzymatic titer as compared to control fed fishes (Adorian *et al.*, 2019) . The higher RBC count contribute towards the health of fish by stimulation of defence system (Nya & Austin, 2009). The increase in WBCs indicate stimulation of innate immune system (Misra *et al.*, 2006a). The hemoglobin content of blood is very important in transporing oxygen to fishes, the increase in its content indicate better health (Talpur & Ikhwanuddin, 2012). A statistically significant correlation was found between fish weight, length and blood parameters studied in two species of fishes (Fazio *et al.*, 2015). Hematology is related with metabolism rate , rise in RBCs, HGB and HCT results in higher metabolism which leads to better growth. HCT showed positive significant relationship with RBCs, MCV and amylase whereas negative significant correlation was found with MCH, MCHC. The increased metabolism will lead to increase in volume of red blood cells consequently HCT value could rise (Jawad *et al.*, 2004). MCV was significantly positive in correlation with HCT, amylase and

glucose and significantly negatively correlated with MCH, MCHC, % growth, FCE and SGR at 45th day of trial while at 90th day hematocrit count showed significant positive relationship with MCV, LYM% and percentage growth rate and non-significant relationship with other parameters. Significantly negative relationship was found between Mean corpuscular volume and MCHC, PLT and LYM% while relationship with other parameters was non-significant. MCH was significantly positively correlated with MCHC, PLT, and negatively correlated with RBC, HCT, MCV, lymphocyte %, amylase and fat. MCHC showed positive correlation with MCH, PLT, % growth and SGR while it showed negative correlation with RBC, HCTs, MCV, FCR, Amylase and glucose. Lymphocyte% is significantly negatively correlated with MCH at 45th day but at 90th day lymphocytes showed non-significant relationship with percentage growth rate, SGR, FCR, FCE, protease and amylase. % growth at 45th day was positively correlated significantly with WBCs, HGB, MCHC, PLT, FCE, SGR and protein and had significant negative correlation with MCH. MCV, FCR, amylase and glucose while at 90th day the correlation analysis showed that % growth rate showed significant positive relationship with main hematological parameters (WBC, RBC, HGB, PLT and LYM %), SGR, FCE and protease activity while significant negative relationship with FCR and amylase. At 90th day platelets count showed significant positive relationship with LYM% while non-significant with rest of parameters. Earlier it was observed that Bacillus supplementation in certain fry's impoved digestion, growth, increased SGR and reduced FCR (Bagheri *et al.*, 2008). FCR showed significant positive relationship with MCV, cellulase, amylase and glucose and showed significant negative relationship with MCHC, % growth, FCE, SGR and protein at 45th day while at 90th day feed conversion ratio showed significant positive relationship with amylase activity, significant negative relationship with FCE and non-significant with protease. FCE showed significant positive relationship with HGB, % growth, SGR, protein and had significant negative correlation with MCV, FCR, cellulase, amylase and glucose at 45th day while at 90th day it was observed that feed conversion efficiency showed significant negative relationship with protease and amylase activity.

Protease was found to significantly negative correlated with WBC and had no significant positive relationship with other parameters undertaken in the study. Cellulase showed significant positive relationship with FCR, amylase and showed significant negative

relationship with FCE and protein while with other factors no significant relationship was found out. Amylase showed significant positive correlation with HCT, MCV, FCR, cellulase and glucose and was significantly negative correlated with MCH, MCHC, % growth, FCE, SGR and protein white it didn't showed any significant relation with the rest of factors. SGR was found to be significantly positively correlated with WBC, HGB, MCHC, PLT, % growth, FCE, and protein and showed significant negative relation with MCV, FCR, amylase and glucose and no significant correlation was found out with other factors at 45th day while at 90th day specific growth rate showed significant negative relationship with amylase while non-significant with FCR, FCE and protease. Protein showed significant positive relation with WBC, HGB, RBCs, % growth, FCE and SGR and significant negative relationship with FCR, cellulase, amylase and glucose while with other factors no significant results were found out. At 90th day there was a significant negative relationship between protease and amylase activity. Fats showed significant negative relationship with MCH while with all other factor no significant relation was found out.

Ash showed no significant relationship with any factor studied during the designed research. Glucose showed significant positive correlation with MCV, FCR, amylase and showed significant negative relation with WBC, HGB, MCHC, PLT, % growth, FCE, SGR and protein while with other factor glucose was not significantly correlated at 45th day of feeding trial (Table 4.12a and 4.12b).

G. candidum QAUGC01 co-culture with *B. cereus* QAUBC02 showed antagonistic activity against *S. aureus* both *in vitro and in vivo conditions.* The fishes fed T7 (co-culture of *G. candidum* QAUGC01 and *B. cereus* QAUBC02) probioitc and control fed fishes were injected with *S. aureus* at the end of ninetyly feeding trial to monitor survival. Mortality was observed in all the control fed fishes while survival rate of T7 fed fishes was hundred percent. An earlier study based on the supplementation with *G. candidum* QAUGC01 in early life cycle of *L. rohita* also showed less mortaliy as compared to control when challenged with *S. aureus* (Ibrar *et al.*, 2017). This resistance to pathogens might be due to innate immune stimulation by probioitcs or this might be due to the reduced epithelium permeability to toxins (Sun & O'Riordan, 2013). Pathogen inhibition by *G.*

candidum QAUGC01 could be attributed to its colonization , competitive exclusion and antimicrobial production (Muroga *et al.*, 1987).

Phase-III Impact of probiotics on fish gut microbiology

Animal gut being highly diversified and complex microbial ecological system vital for the homeostasis of the host had always attracted scientists. Intestinal microbiology was investigated by many researchers to understand the quality of water, disease protection, meat quality, potential health risks to sea food consumers (Gonzalez *et al.*, 1999; Shelby *et al.*, 2006). Gut microbiota plays a pivotal role in controlling the overall physiology of fishes (Sekirov *et al.*, 2010). Gut microbiology can be examined both by culture dependent and independent techniques. In the present study both approaches were used. Different microbial groups were quantified such as Lactic acid bacteria (MRS), yeasts (OGA), Gram negatives (MacConkey), *Enterococcus* and *Streptococcus* (M17) and total aerobic, TABC bacterial count (TSA). The range of total bacterial count recorded from 1.92×10^6 – 1.01×10^8 CFU/g, highest bacterial count was observed in T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) was 1.01×10^8 which was significantly higher than control. The level of presumed lactic acid bacteria (MRS) ranged from 1.52×10^6 – 9.92×10^7. T1 group fed with *G. candidum* QAUGC01 showed highest aerobic count as well as LABs count. Our results are similar to earlier report that higher aerobic and LABs count was found in *Red Tilapia* fed with *Pediococcus acidilactici* supplemented diet at the concentration of 10^7 CFU/g (Ferguson *et al.*, 2010). Some treated groups showed lower count than control lowest aerobic value observed in T5 fed on (*G. candidum* QAUGC01 and *E. faecium* QAUEF01 *co-culture*) was 1.92×10^5 cfu/g, while minimum LABs count was observed in T3 (*E. hirae* QAUEH01) 1.52×10^6 cfu/gm. The reason might be lower number of cultivable bacteria in samples, improper growth media or inappropriate conditions for communities. Lower LABs count might be due to the fact that Lactic acid bacteria are not the dominant microflora of fish gut (Ringø *et al.*, 1998). Assumed *Enterococcus* and *Streptococcus* count range was recorded between 5.00×10^5 – 2.87×10^8 cfu/gm. Highest count was recorded in T7 group fed with *G. candidum* QAUGC01 and *B. cereus* QAUBC02 co-culture and lowest in T3 fed (*E. hirae* QAUEH01).

Assumed Enterobacteriace or coliforms count ranged from 1.18×10^6 – 1.64×10^7. All probiotic treated groups showed lower Coliforms count as compared to control fed with

basal diet. These findings are in accordance with reports that feeding *O. niloticus* with dead *Saccharomyces cerevisie* and *Bacillus subtilis and Saccharomyces cerevisie* combination live significantly reduce the Coliforms such as (*E. coli, Salmonella, Klebsiella*) count as compared to the control fed with basal diet (Marzouk *et al.*, 2008). Yeast count of the groups was found between $3.33\times10^5 - 8.41\times10^7$. Highest yeast count was observed in T1 group fed with *G. candidum* (QAUGC01) and lowest was in T5 fed with *G. candidum* QAUGC01 and *E. faecium* QAUEF01 co-culture.

In present study metagenomics method was used to evaluate the intestinal microbiome after the 90th days probiotics feeding trial, is scientific approaches provide detailed information regarding intestinal microbial communities. The sequencing centers shifted from time consuming Sanger method to cost effective and efficient next generation technologies to explore diversified intestinal communities (Wetterstrand, 2016). The advancement of next generation technologies enable us to understand genome of whole microbial community in a sample (Roumpeka *et al.*, 2017). The research on fish gut communities and their manipulation to get healthy and productive aquaculture is in initial stage (Montalban-Arques *et al.*, 2015). The increase in growth of fishes by probiotic administrated is linked with its manipulation of gut microbial community (Carnevali *et al.*, 2017). The health and welfare of fish is correlated with the balanced microbial community which is designed by both the environment and host mediated factors (Lokesh *et al.*, 2019).

In current study administration of the *G. candidum* QAUGC01 and *B. cereus* QAUBC02 in consortium reduced intestinal microbial communities in terms of specie richness, same reduction was reported by studies conducted by (Bakke-McKellep *et al.*, 2007; Geraylou *et al.*, 2013; Ringø *et al.*, 1998). It was reported that application of *L. lacti s*ssp. *lactis*ST G45 and AXOS and *B. circulans* ST M53 and AXOS in combination with feed resulted in a significant reduction in the gut bacterial diversity of Siberian sturgeon (Geraylou *et al.*, 2013). In the present study most abundant phyla was *Proteobacteria* which is in accordance with the previous findings relating with the dominance Proteobacteria phyla in carp intestine (Desai *et al.*, 2012). Proteobacteria is the most dominating community found in the intestinal tract of many freshwater fishes (Lokesh *et al.*, 2019). Proteobacteria in fishes is involved in several metabolism pathways, stress reduction (Vikram *et al.*, 2016). This phylum is involved in digestion as well (Romero *et al.*, 2014).Contradictory findings were

also found stating that *Firmicutes* and *Bacteroidetes* are dominant phyla in vertebrates and mammalian gut, the change in fish gut microflora might be due to difference in habitat, seasons, geographic, diet, type of species and water quality (Ley *et al.*, 2008). Treated group showed presence of *Bacillus*, lactic acid bacteria and *Enterococcus* also but there percentage was low.

Proteobacteria represented the first dominant OTU in all groups except T2 (*E. faecium* QAUEF01), while in T0 basal diet taken as control and T4 (*B. cereus* QAUBC02) γ class and in T1 (*G. candidum* QAUGC01) and T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 β class of *Proteobacteria* was dominated. The shared and unique general was found among all the dietary treatments in both fungal and bacterial metagenomics. The shared sequences might represent the core microbiota . In an earlier study 46 (genus/specie) OTUs were shared among all the dietary groups in teleost fish. These shared belonged to core phylum Proteobacteria (60%), Bacteroidetes (17.4%) and Firmicutes (8.7%) (Piazzon *et al.*, 2017). The unique OTUs might be due to the differences in the dietary supplements (probiotics treatments) in our trial. It is a established fact that more than 50% of variation is gut microbiota is diet related (Abruzzo *et al.*, 2016). The present research showed that the *E. faecium* QAUEF01 fed groups had a shown a significant decline in Proteobateria. An overall increase in Firmicutes were observed, the Proteobacteria observed in this group was reduced to (0.872862%) and the percentage of Firmicutes were (99.04808 %). Similar results were elucidated in a study in which fish isolated lactic acid bacteria modulated intestinal community structure of Atlantic salmon. This modulation might be due to the change in microbial association caused by the lactic acid bacteria. The driving factors responsible for this modulations needs to be further investigated (Gupta *et al.*, 2019).Our results were similar to the previous findings which stated that the *Proteobacteria* and *Firmicutes* are most abundant and *Bacteroidetes* are relatively less in the intestinal flora of most of the fishes (Roeselers *et al.*, 2011). *Actinobacteria* basically produce secondary metabolites especially antibiotics which might be effective in controlling pathogens, in all our treatments used in trials. Actinobacteria was present which might be responsible for keeping fish healthy by production of antibiotics.

Achromobacter was higher in treated group T1 (*G. candidum* QAUGC01), T5 (*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) and T7 (*G. candidum* QAUGC01 co-culture with *B. cereus* QAUBC02) which is common in marine environment and is an indicator of aquatic environment. Metagenomics data revealed that *Staphylococcus saprophyticus*, a fish pathogen was detected in control fed groups but in probiotic fed fishes either it was absent such as treatment T1 (*G. candidum* QAUGC01) and T5(*G. candidum* QAUGC01 co-culture with *E. faecium* QAUEF01) and in rest of treatment it was in very low percentage which might be due the production of antimicrobial by probiotics or competitive exclusion. It is indicating that probiotics have modulated the gut microflora by either reducing their number to undetectable level or by production of antimicrobials. This is inline with our previous in vitro tests for antipathogenic activity in which *G. candidum* QAUGC01 was effective against all the tested pathogens and *B. cereus* QAUBC02 was also effective aginst some of the tested pathogens(Naz *et al.*, 2013). It was reported in an earlier study dietary administration of probiotic *B. pumilus* modulated the gut microbiota of *E. coioides* by boosting healthy bacteria and reducing the population of potential pathogen *Staphylococcus saprophyticus* (Sun *et al.*, 2011). In the present study *Clostridium* and *Ruminococcus spp*. were present conjugating the dietary habits of *L. rohita*. Our results corroborate with the earlier studies done on microbal diversity in *L. rohita* (Flint *et al.*, 2012; Singh, 2017). Pathogenic bacteria and yeast such as *Mycobacterium, Clostridium, Staphylococcus, Yersinia, Shigella, Cryptococcus, E. coli, A. hydrophilla, Vibrio* were completely absent in the probiotic fed group. This is in agreement with the earlier findings in which *Lactobacillus rhamnosus* applied as probiotic in *C. carpio* control *A. hydrophila* infection (Harikrishnan *et al.*, 2010a). In the present study *Enterococcus* species and *Bacillus* speices used as probiotics in T2 (*E. faecium* QAUEF01) and T4 (*B. cereus* QAUBC02) treatments were detected in low percentages which might be due to the their low adhesive property in live fish systems. This might also be their inability to proliferate substantially for ninety days due to host related or environmental factors.

Moreover due to the change in community structure and their relative abundance it is inferred that the modulation is mediated by dietary supplementation. A previous study based on study of intestinal communites in fish supplemented with *Lactobacillus* sp and

Enterococcus sp showed that these bacteria were not revealed in metagenomics data which might be their very low abundance failed ot detected and other reason could be low adhesion ability (Sha *et al.*, 2016). Treated groups T1 (*G. candidum* QAUGC01), T5 (*G. candidum* QAUGC01co-culture with *E. faecium* QAUEF01) and T7 (*G. candidum* QAUGC01co-culture with *B. cereus* QAUBC02) showed high *Galactomyces* count *such as Galactomyces candidum, Galactomyces geotrichum, Galactomyces* species as compared to control which showed higher count of *Debaryomyces hansenii* 88.61%. *Glactomyces* presence showed that administered *G. candidum* QAUGC01 survived and persisted in the gut of *L. rohita* fish and modulate the gut microbiology. Previous studies conducted by reported that the yeast was well established in larvae Zebrafish gut when fishes fed it as probiotics (Caruffo *et al.*, 2015; Field *et al.*, 2009; Tovar-Ramırez *et al.*, 2004). It was reported that fish growth rate was significantly increased due to yeast supplementation and persistence in fish gut (Tovar-Ramırez *et al.*, 2004). It was found that the Zebrafish which fed on native gut yeast as probiotics showed increased survival when challenged by *Vibrio anguillarum* (Caruffo *et al.*, 2015). The yeast species have better adaptability and survival in gut thereby increasing immunity and protect against disease by modulation of gut immunity. *Sporobolomyces lactosus* was absent in all the treatments used in research which is an opportunistic pathogens and rarely caused disease in immune-deficient fishes and poor environmental conditions as reported earlier (Galuppi *et al.*, 2001). Similarly other opportunistic yeast pathognes such as *Trichosporon* and *Cryptococcus* were found in low percentage in control and treated groups. Certain yeast were reported to produce polyamines which have strong affinity to adhere to mucous epithelium and colonization our results supports this finding as *G. candidum* QAUGC01 was retrieved in yeast metagenomics data which might be due to the production of polyamines by them that facilitated in adherence and consequent persistance (Andlid *et al.*, 1995). The principal component analysis showed that T1 (*G. candidum* QAUGC01) fed group showed significant positive correlation among specific growth rate, protein content, protease and hematocrit. All these factors are interlinked contributing to the overall increase in metabolism and digestivity thus enhancing growth and activity of fishes. This probiotic has modulated the gut microflora in the terms of absence of potential pathogens of fishes like *Mycobacterium* and *Staphylococcus* sp as compared to control fed group. It

might be due to exclusion principal or due to production of antimicrobial compound by *G. candidum* QAUGC01 (Naz *et al.*, 2013). The mechamism of modulation needs to be further explored. Moreover it has enhanced the level the health promoting genera such as *Enterococcus* , *Bacillus*, *Achromobacter*, and *Paenibacillus* thus modulation might be a driving force and connectivity towards better physiological parameters. This probiotic supplementation has promoted health of the fish by modulating the gut microflora. Our results are strongly supported by previous research based on the modulation of gut microbiota by supplementation of yeast extract which resulted in better growth and health of fish by increasing the relative abundance of beneficial bacteria (Liu *et al.*, 2018). A number of earlier findings had validated the fact that yeast dietary supplements improved Nile tilapia heath by improving digestibility and blood immunological status (Berto *et al.*, 2016). According to Pco A there is significant correlation between control group and glucose level while all the probiotic based treatments has shown less glucose level. The probiotic treatment T7 (*G. candidum* QAUGC01co-culture with *B. cereus* QAUBC02 showed significantly positive correlation with RBCs, HGB and WBCs . The improved physiology might be the outcome of increase in relative abundance of benefical microbes and their interaction with host . Commercial probiotics showed relatively high number of pathogens such as *Salmonella enterica*, *Klebsiella oxytoca, Serratia quinivorans*. The *G. candidum* QAUGC01 was an indigenous isolate from yougart which has enhanced growth both in single form and co-culture form. *G. candidum* QAUGC01 have executed synergistic effects on *B.cereus* QAUBC02 . This combination had significant improvement in fish physiology which might be due to shift in microbial population toward a balanced microflora. The low percentage of *Bacillus* in T7 (*G. candidum* QAUGC01co-culture with *B. cereus* QAUBC02) treatments might be due to numerous environmental and host related factors. The interactions between microbes remains complex and need further evaluation by using more molecular analysis of metabolic pathway activated by probiotics.

The present study revealed the presence of array of metabolic, stress and regulatory peptides from intestinal tract of *G. candidum* QAUGC01 fed fishes based on Maldi/TOF/TOF analysis. A variety of regulatory proteins were found in proteomic analysis of fish intestine fed on *G. candidum* QAUGC01. These proteins were controlling the cellular machinery by regulating transcription process. This included mitochondrial

malate which is key enzyme associated with aerobic cellular energy production. Moreover it is also associated with other metabolic function like gluconeogenesis and lipogenesis (Labrou & Clonis, 1997; Takahashi-Íñiguez *et al.*, 2016). Proteomic analysis also revealed the presence of multiple stress proteins. As the fishes are continuously exposed to various abiotic and biotic stresses these stress proteins helps to enhance the survival of fishes in stress conditions (Barton, 2011). The present study showed the presence of stress proteins such as thiorexodin peroxidase that prevent cell collapse from reactive oxygen species in oxidative stress. Earlier findings had shown the enzyme thiorexodin peroxidase isolated from Chinese fly prevent it from environmental stresses (Huaxia *et al.*, 2015; Yao *et al.*, 2013). Carbohydrates serves as an important source of herbivorous fishes such as *L. rohita* and the presence of carbohydrate metabolic enzymes in *G. candidum* QAUGC01 peptides validated this fact. *L. rohita* can utilize up to 43% carbohydrate without any adverse effects on health (Kumar *et al.*, 2010). These proteins thus helped the fish to be metabolically active prevent them from different stresses and regulated the transcription for better growth and survival.

CONCLUSION

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It could be concluded from present study that in combination microbial strains were able to survive better in simulated gut conditions. Moreover, mix-culture application in fish feed comparatively improved fish growth, physiology, health and nutritional profile. Non-existence of pathogens in gut and improved enzymatic titer in probiotic fed fishes leads to better digestion and supreme health. Probiotic modulated gut community by strengthen the composition and number of healthy nonpathogenic microflora which is crucial for wellbeing of host. Furthermore it is important to understand the underlying molecular mechanisms that participate in interacting the probiotic with resident gut communities.

It was concluded that fish intestinal tract harbored diverse microbial community based on culturing methods. Variable probiotic potential was exhibited by the strains used in the present study. The present study showed that the probiotics have altered the modulated the microbial community which is associated with improved physiology. The strains from different sources showed variable potential of probiotics. *E. faecium* QAUEF01 either as monostrain or consortium with *G. candidum* QAUGC01was proved to be an effective probiotic as it was found to be acid tolerant, bile tolerant, exhibited good hydrophobicity Fish isolate *B. cereus* QAUBC02 performed well both in vitro and in vivo conditions. It enhanced the enzymatic levels of cellulase and amylase significantly.

The applied probiotics affected the overall physiology of fish such as growth, weight, enzyme production, enzymatic quantity and body composition. T7 (*G. candidum* QAUGC01 and *B. cereus* QAUBC02 coculture) was found to be more efficient in enhancing growth, RBCs, and hematocrit. This combination needs further validation tests in future studies. This combination also enable the fishes to survive when they are injected with *Staphylococcus aureus*. Combination of probiotics delivered better results as compared to single strains used as probiotics. *B. cereus* QAUBC02 (T4) treatment were found to be potential amylase and cellulase producer.

The core phylum found in all the treatments include Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Probiotics used in trial modulated microbial community by changing the level of microbial population. The metagenomic analysis of fish intestinal microbial community validated the persistence of G. *candidum* QAUGC01.

The treatments based on indigenous probiotics were effective in reducing the potential pathogens such as *Staphylococcus saprophytic* and beneficial bacteria number rise such as *Achromobacter* which eliminate the nitrogenous waste and maintain water quality for better fish Cultivation. Opportunistic yeast as *Trichosporon* and *Cryptococcus* were also found in low percentage in control and treated groups

FUTURE PROPECTS

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In future additional studies can be conducted for profiling of gut microbiota by using whole genome or functional Metagenomics, Transcriptomics and Proteomics approach. The underlying mechanisms of probiotics action should be investigated. There is need to investigate the profiling of host-microbe interaction, interactions between probiotic microorganisms their resulted co aggregation as well as with other gut microbiota to inhibit and compete with pathogens. Well-designed studies are required to improve the technicality of probiotics concept by exploring the fish pathogens invasion site, proliferation method, virulence factors specifically, so that it can be decided that either water born or food born vehicle would be appropriate for probiotic. As probiotics consortium/ combination is acquiring more attention in recent days, so studies for the development and understanding of this dimension is crucial. After further validation and optimization, the best combination of probiotic strains can be commercialized for the betterment and development of local aquaculture industry.

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APPENDIX

Appendix